

Molecular Genetics
of X-linked Immunodeficiency Disorders

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Declaration

I began working at the Institute of Child Health in July 1985 as a registrar in the Department of Genetics. The publication of Cohen's work demonstrating a family of genes, differentially expressed in lymphocytes, on the mouse X chromosome provoked much discussion between myself, Dr Sue Malcolm, and Dr Robin Callard and Professor Levinsky in the Department of Immunology. We postulated that the X-linked immunodeficiencies affecting the lymphoid lineage in man could be due to abnormalities in the human equivalent of this gene family. We, therefore, started to collect families to map the X-linked immunodeficiencies as a step towards isolating this gene family in man. Dr Yu Lung Lau joined the Immunology Department as a research fellow in January 1986 to collect families with Bruton's X-linked agammaglobulinaemia and I became a research fellow on the project in January 1987. Pooling our data with groups in Paris and Strasbourg we quickly found that Bruton's agammaglobulinaemia mapped to Xq21 and that X-linked severe combined immunodeficiency mapped to Xq13. It rapidly became clear that the localisations of the human X-linked immunodeficiencies did not cluster on one part of the X chromosome.

Having established linkage for X-linked agammaglobulinaemia and X-linked severe combined immunodeficiency we wanted to develop strategies for isolating the defective genes. I felt that the first step in this was to refine their localisation. In order to do this I set out to order known probes on the long arm of the X to act as a framework for mapping the diseases more precisely and also started to build up a physical map of the probes at Xq21 using pulsed field gel electrophoresis. After I had established pulsed field gel electrophoresis in the laboratory I taught the techniques to M O'Reilly who then developed this side of the project. The pulsed field gel electrophoresis results I have described in this thesis precede the time when M O'Reilly took over this aspect of the work.

In the meantime I had found that counselling the families with severe combined immunodeficiency was complicated by the fact that there are X-linked and recessive forms. This made it extremely difficult to counsel couples who had only one affected son and no previous family history of the disease. I, therefore,

Abstract

Eight disorders of the immune system have been described which are inherited as X-linked recessive conditions. The aim of this study has been to improve predictive testing in X-linked agammaglobulinaemia (XLA) and X-linked severe combined immunodeficiency (XSCID) and to investigate the underlying defect in XSCID.

Precise genetic localisation is essential for accurate predictive testing and in order to develop strategies to clone the genes. Before disease localisation can be improved it is necessary to clarify the order of a number of probes within the region of interest. Fourteen genetic markers assigned to the X chromosome between the pericentromeric region and Xq22 were ordered by family studies and deletion mapping. Pulsed field gel electrophoresis was used to make a physical map of the markers linked to XLA.

Knowledge of the order of these anonymous DNA probes led to finding additional linked probes for both diseases. This makes predictive testing possible in more families.

There are X-linked and autosomal recessive forms of severe combined immunodeficiency. This has caused difficulties in counselling couples who have an affected male child and where there is no previous family history of the disease. In this study it has been shown that female carriers of the X-linked disorder have non-random use of the X chromosome in T lymphocytes. This provides a means of distinguishing between the autosomal and X-linked forms which enables more accurate genetic counselling.

XSCID has been mapped to Xq11-q13 using DNA markers which detect polymorphic variation (de Saint Basile et al, 1987). No recombinations have been observed between the disease locus and the anonymous DNA probe cpX289. In this study the PGK1 locus was also shown to be closely linked to the disease. Using both of these linked markers predictive tests can be offered to 65% of families.

The probe pSPT/PGK which detects a polymorphism at the PGK1 locus can also be used to investigate X chromosome usage. In females who are heterozygous for the polymorphism detected by this probe, carrier detection and assignment of phase can be carried out in the same procedure. This is a unique situation and is particularly useful when the proband could carry a new mutation or when there are no males available who can be used to assign linkage phase.

It has been thought that XSCID results from a defect in a T lymphocyte specific gene because the phenotype is predominantly a lack of T lymphocytes and because host B lymphocytes produce functional antibody following transplantation and engraftment of T lymphocytes. Finding a non-random pattern of X chromosome usage in a mature cell population implies that the defective gene is expressed in that cell type and this technique was used to investigate gene expression. Non-random X chromosome usage was found in T lymphocytes, B lymphocytes, monocytes and granulocytes. The pattern of expression suggests that the underlying defect in XSCID is in a general metabolic pathway rather than a pathway specific to lymphocytes.

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CHAPTER 1. Introduction: X-linked Immunodeficiencies

Eight X-linked disorders of the immune system have been described (Table 1.1). These disorders affect different aspects of the immune system. Six affect the lymphoid system, though neither the immunoglobulin genes nor the T-cell receptor genes are on the X chromosome. They are X-linked agammaglobulinaemia, X-linked severe combined immunodeficiency, X-linked hyperimmunoglobulinaemia M, X-linked lymphoproliferative syndrome, Wiskott-Aldrich syndrome and X-linked agammaglobulinaemia with growth hormone deficiency. The remaining X-linked immunodeficiencies are chronic granulomatous disease and properdin deficiency. Chronic granulomatous disease is a defect of phagocytosis due to absence of the β chain of cytochrome b_{-245} (Teahan et al, 1987). Properdin is a regulator in the alternative pathway of complement and its deficiency is associated with meningococcal infections (Sjoholm et al, 1982).

X-linked severe combined immunodeficiency
X-linked agammaglobulinaemia
X-linked hyperimmunoglobulinaemia M
X-linked lymphoproliferative disease
X-linked agammaglobulinaemia with growth hormone deficiency
Wiskott-Aldrich syndrome
X-linked chronic granulomatous disease
Properdin deficiency

Table 1.1 X-linked Immunodeficiencies

1.1 Localisation of the X-linked Immunodeficiencies involving the Lymphoid System

As six diseases involving the lymphoid system are X-linked it has been suggested that there are regulatory genes involved in lymphocyte development on the X chromosome. Cohen and his colleagues isolated a murine X-linked cDNA clone that is developmentally expressed in lymphocytes but not expressed in macrophages, liver cells or kidney cells (Cohen et al, 1985a; Cohen et al, 1985b). This clone, XLR (X-linked, lymphocyte regulated), detects a family of genes on the mouse X chromosome.

CBA/N xid mice carry an X-linked mutation which renders them incapable of producing antibodies to soluble polysaccharide (Scher, 1982). It has been shown that the XLR gene family is closely linked to the X-linked immunodeficiency locus in the xid CBA/N mouse strain (Cohen et al, 1985a).

There is, therefore, an X-linked gene family in the mouse which is differentially expressed in B and T lymphocytes and is linked to an immunodeficiency disorder in a mutant mouse strain. This raised the possibility that the lymphoid X-linked immunodeficiencies in man could be due to mutations in different genes in a conserved gene family. However, five of the six X-linked lymphoid disorders have now been localised and all map to different regions of the X chromosome (figure 1.1).

X-linked severe combined immunodeficiency (XSCID) has been localised to Xq11-q13 (de Saint Basile et al, 1987).

X-linked agammaglobulinaemia (XLA) has been mapped to Xq21.3-22 (Malcolm et al, 1987). X-linked hyperimmunoglobulinaemia M, in which IgA and IgG are absent as in XLA but IgM levels are normal or elevated, does not map to the same region of the X chromosome as XLA (Malcolm et al, 1987) and there has been a report suggesting linkage to Xq24-q27 (Mensink et al, 1987). X-linked agammaglobulinaemia with growth hormone deficiency has only been described in one family (Fleisher et al, 1980). The disorder in this family could either be a single entity or two X-linked disorders segregating together, although there have been no reports of isolated growth hormone deficiency being transmitted as an X-linked recessive condition. There are recombinations in this family between the disease and a probe, S21, which maps to Xq21.3-22 (Notarangelo et al, 1988).

Individuals with X-linked lymphoproliferative syndrome are unable to mount an appropriate response to Epstein-Barr virus infection. Before EBV infection affected males have normal cellular and humoral immune responses. After EBV infection 75% develop fatal infectious mononucleosis. Those that survive have defects in cellular and humoral immunity and a high incidence of lymphoma. Linkage studies have demonstrated significant linkage to probes mapped to Xq24-q27 (Skare et al, 1987).

Wiskott-Aldrich syndrome is characterised by impaired cellular and humoral immunity, thrombocytopenia and eczema with an increased incidence of lymphoreticular malignancies. This condition has been mapped to Xcen-Xp11.3 (Peacocke and Siminovitch, 1987; Kwan et al, 1988).

1.2 Severe Combined Immunodeficiency

Severe combined immunodeficiency (SCID) is a group of disorders that can be inherited in X-linked or autosomal recessive forms. The disease is characterised by absence of cellular and humoral immunity. Affected infants have reduced numbers of T cells, no proliferative response to mitogens, and hypogammaglobulinaemia although B cell numbers may be normal or elevated.

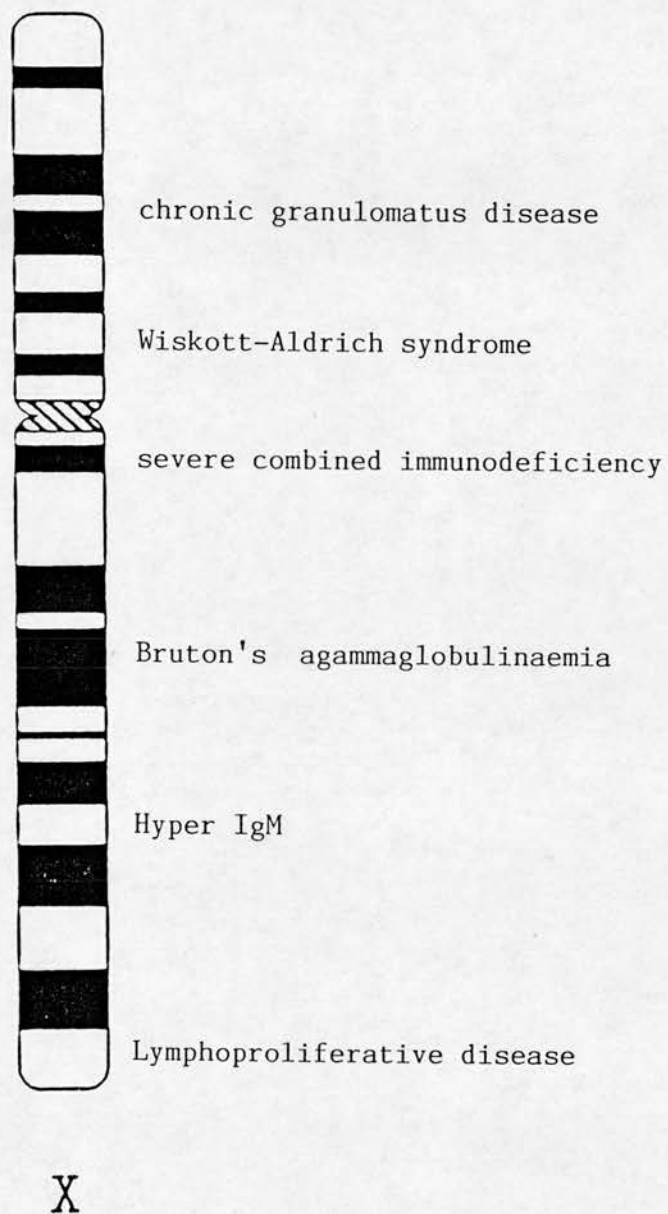


Figure 1.1 The localisation of X-linked Immunodeficiency Disorders on the X Chromosome

Infants present within the first six months of life with failure to thrive, oral candidiasis refractory to treatment, recurrent infections and intractable diarrhoea which is often secondary to one of the enteric viruses. Pulmonary infections with pneumocystis carinii, cytomegalovirus, measles virus or parainfluenza virus are usually fatal. Chronic hepatitis may follow cytomegalovirus infection and a variant of sclerosing cholangitis with focal disappearance of bile ducts is also seen.

The incidence of the disease has been estimated to be 1 in 70,000 live births (Fath, 1982). Without bone marrow transplantation it is fatal within the first few years of life. There is 90% survival following a human leucocyte antigen (HLA) matched bone marrow transplant but only 50% survival after a mismatched transplant (Fischer et al, 1986). A number of methods of depleting T cells in the donor marrow have been tried to prevent graft-versus-host disease (Reinherz et al, 1982; Morgan et al, 1986).

SCID is a heterogeneous group of diseases. Adenosine deaminase deficiency accounts for half of the autosomal recessive cases. Deficiency of purine nucleoside phosphorylase, another enzyme in the purine degradation pathway, also causes SCID. Reticular dysgenesis can be differentiated from SCID by the absence of granulocytes and bare lymphocyte syndrome can be differentiated from XSCID by the absence of expression of MHC class II genes. After excluding these conditions the remaining group includes both autosomal and X-linked recessive cases which cannot be distinguished clinically or using immunological or biochemical tests. Obligate female carriers of XSCID do not have any known biochemical or immunological abnormalities.

The male to female ratio of children with SCID is 4:1 and therefore 60% of all cases will be X-linked. 75% of sporadic males will have the X-linked disease and as one third of these will be the result of a new mutation 50% of the mothers of sporadic males will be carriers of the X-linked disorder. Where the two enzyme deficiencies have been excluded the proportion of sporadic males who have the X-linked form will be 6/7 (86%) and two thirds of their mothers will be carriers of the disease. Female carriers of XSCID do not have any known biochemical or immunological abnormalities. Genetic counselling would be greatly improved if these women could be identified.

The X-linked form of the disease has been mapped to Xq11-q13 (de Saint Basile et al, 1987) and in families with XSCID both carrier detection and prenatal diagnosis can be offered using linked DNA probes. In those families where the inheritance pattern is not clear prenatal diagnosis can still be offered, if the abnormality in the affected child was well defined, by sampling fetal blood in the mid trimester (Linch et al, 1982).

A number of observations have led to the theory that the primary defect in XSCID is in the T cell series. The phenotype in XSCID is predominantly a lack of mature circulating T cells with normal or high B cell numbers. In vitro work suggests that the B cells function in the presence of normal T cells (Seeger et al, 1976; Pahwa et al, 1980) and that the B cells proliferate in response to T cell independent mitogens, such as *Staphylococcus aureus* Cowan I (Schuurman et al, 1980). However, details of family history are not given in the papers describing these results and so it is not certain that these findings apply to XSCID. A further piece of evidence that XSCID is a T cell defect is that full cooperation has been observed between donor T cells and host B cells to produce functional antibodies following HLA identical bone marrow transplantation where only the T cells have engrafted (Griscelli et al, 1978). The results presented in this thesis, however, show that the gene defective in XSCID is expressed in other haematopoietic cells in addition to T cells.

1.3 X-linked Agammaglobulinaemia

XLA was first described by Bruton in 1952 (Bruton, 1952a; Bruton et al, 1952b). Affected males present in the second half of the first year of life as maternal antibody levels start to decline. Lederman and Winkelstein have carried out a survey of 96 affected males. In this series recurrent sinopulmonary infections were one of the presenting features in 84 cases, gastrointestinal infections in 34 cases and mono- or oligoarthritis in 19 cases (Lederman and Winkelstein, 1985). The causative organisms in the arthritis included bacteria, viruses, and atypical organisms such as *Mycoplasma pneumoniae* and *Ureaplasma urealyticum*. Vaccination with attenuated live poliovirus has caused paralytic poliomyelitis and no live vaccine should be given to patients with XLA. *Giardia* infection is a recognised cause of chronic diarrhoea and malabsorption in affected males. The major cause of morbidity in the series of patients reviewed by Lederman and Winkelstein was chronic lung disease. The two main causes of death were cardiorespiratory failure, resulting from chronic pulmonary disease and cor pulmonale, and disseminated viral infection. The age at death ranged from four years to twenty eight years. The oldest survivor in the series was twenty six years. It is difficult to assess the outlook of patients presenting now as in recent years the diagnosis has been made at an earlier age and treatment with immunoglobulin replacement started earlier.

The only estimate of the incidence of XLA comes from the Australian national register (Hosking and Robertson, 1983). This gives an incidence of 1 in 500,000 but may not reflect the incidence in other populations.

The disease is characterized by low levels of all serum

immunoglobulins and absence of functional antibodies but normal cell-mediated immunity (WHO Report, 1983). Carrier females have normal serum levels of immunoglobulins.

Obligate female carriers do not have any detectable abnormalities and there is no immunological or biochemical carrier test. Neither is there a reliable immunological method for prenatal diagnosis as absence of B cells has been observed in normal fetuses at 20 weeks gestation (Linch and Levinsky, unpublished results). Conversely the presence of B cells in fetal blood does not entirely exclude the possibility of an affected male as variation in B cell numbers has been described (Leickley and Buckley, 1986). Investigation of the B cell series, therefore, cannot be used for prenatal diagnosis.

The first linkage study of XLA suggested localisation to Xq21.3-22 with a maximum lod score of 2.17 at $\theta=0$ with S21 and a maximum lod score of 3.65 at $\theta=0.04$ with 19.2 (Kwan et al, 1986). A linkage study reported shortly after this localisation suggested that there might be non-allelic heterogeneity (Mensink et al, 1986). A third study supported the findings of Kwan with maximum lod scores of 6.6 with pXG12 and 4.4 with S21, both at $\theta=0$ (Malcolm et al, 1987). The possibility of non-allelic heterogeneity is very important if these probes are to be used clinically. A method has been described to allow for non-allelic heterogeneity in risk calculations using the probes S21 and pXG12 (Lau et al, 1988).

The disorder in XLA is confined to the B cell series. There are few or no circulating surface immunoglobulin positive B lymphocytes but pre-B cells are found in normal numbers in the bone marrow (Pearl et al, 1978) suggesting that the basic defect affects the maturation of pre-B cells. However, the failure of differentiation from pre-B cells to B cells is not absolute as small numbers of surface IgM-positive B cells are found in the peripheral circulation (Conley, 1985). There are reports of phenotypic heterogeneity between patients from different families (Golay, Webster, 1986; Dobozy et al, 1986) and between patients within families. Thus, in large X-linked pedigrees, some affected males have cells with surface IgM and IgD and cells secreting IgM, whilst other affected males in the same family have no circulating B cells (Leickley and Buckley, 1986). These studies suggest the primary defect in XLA may affect B cells at more than one stage of development. X inactivation analysis has shown that the defect is intrinsic to the B cell series rather than lack of an extrinsic factor (Conley et al, 1986). Cooper has proposed that XLA could be due to a rate limiting defect in the gene rearrangement process (Cooper et al, 1986) and this would be consistent with finding a few cells which express surface immunoglobulin or secrete immunoglobulin in some affected individuals.

CHAPTER 2. Introduction: Genetic Map of the X Chromosome

The haploid human genome contains about 3×10^9 DNA basepairs and is estimated to be 3000 centiMorgans long. The X chromosome is approximately 200cM long (Drayna and White, 1985; Donis-Keller et al, 1987). When data was collated at the ninth Human Gene Mapping Meeting there were 32 cloned genes on the X chromosome and 273 anonymous cloned DNA segments. Restriction fragment length polymorphisms were detected by 11 genes and 114 of the anonymous DNA fragments, giving a total of 125 RFLPs on the X chromosome. As the human X is 200cM long if the probes were evenly spaced then there would be an RFLP every 1.6cM and it should be possible to map every X-linked trait.

2.1 Linkage Analysis

2.1.1 Background

Mendel's studies of single clearly defined pairs of characters in the garden pea, presented in 1865, led him to the conclusion that inherited characteristics are determined by pairs of hereditary factors and that the two members of a single pair of genes pass to different gametes during reproduction. He also observed that each pair of characteristics segregated independently from each other pair.

Chromosomes were first described by Flemming in 1877. Reduction division, meiosis, was described in the 1880s and 1890s. Sutton, a student at Columbia University recognised the association between Mendel's inherited factors and the segregation and assortment of homologous chromosomes at meiosis in 1902. He suggested that factors segregate independently when carried on different chromosomes but that factors carried on the same chromosome would be inherited together i.e. show linkage. As there are more independently inherited characteristics than chromosomes de Vries suggested in 1903 that exchange occurs between paired homologous chromosomes at meiosis.

The first linkage between characteristics was found in the vetch, *Lathyrus odoratus*, by Bateson, Saunders and Punnett in 1908. If the characteristics were A, with loci A and a, and B, with loci B and b, then an individual who has received AB from one parent and ab from the other parent would be expected to have gametes carrying AB, ab, Ab and aB in equal numbers according to the law of independent segregation. However Bateson and his colleagues found that some combinations were observed more frequently and others less frequently than expected. When more progeny are found to inherit AB and ab than Ab or aB then the markers are said to be linked.

Sturtevant, an undergraduate in T.H.Morgan's laboratory, suggested in 1913 that genes are arranged linearly on chromosomes and that the proportion of crossovers between two loci on the same chromosome is a measure of the distance between them. The term

recombination fraction was introduced to describe the proportion of recombinants in the total number of offspring and, therefore, the genetic distance between the two loci. Sturtevant proposed that the unit distance over which a cross-over would occur in every gamete be termed the Morgan (M) and hence the distance over which one would expect to see a recombinant in one out of a hundred gametes a centiMorgan (cM).

Recombination fraction and distance in centiMorgans are directly proportional up to distances of 20cM. If A, B, and C are three loci on a chromosome, the distance between loci A and C should equal the sum of the distances AB and BC (when B lies between A and C). This relationship no longer holds for distances greater than 20cM. Sturtevant suggested that finding the recombination distance AC less than AB plus BC could be explained by the occurrence of double crossovers.

2.1.2 DNA Polymorphisms

Restriction endonucleases cleave DNA at specific nucleotide sequences. Neutral single base differences are found approximately every 200bp in genomic DNA. When these abolish or create restriction endonuclease sites digestion with that enzyme will generate two distinct lengths of DNA. Such alterations are called restriction fragment length polymorphisms (RFLPs). The first human RFLP was detected 3' of the β globin chain by an enzyme from *Haemophilus parainfluenza* (HpaI) (Kan and Dozy, 1978). The potential use of these polymorphisms as markers for mapping was pointed out by Botstein (Botstein et al, 1980). It has been noted that the X chromosome has several fold lower density of polymorphism than the autosomes (Hofker et al, 1986).

A second type of restriction fragment length polymorphism results from length variation between two restriction sites. This length variation is usually due to a nucleotide sequence being repeated a variable number of times in tandem and they are referred to as VNTRs (variable number of tandem repeats). This type of polymorphism generates more than two fragment lengths or alleles.

In linkage studies meioses only provide information if it is possible to differentiate between the two chromosomes at the two loci being considered. Using the type of RFLP generated by a single base change at most only 50% of meioses will be informative. From the Hardy-Weinberg equation it can be calculated that when the restriction site is present on 50% of the chromosomes in the population then the number of informative individuals, $2pq$, is 50%. When RFLPs result from tandem repeats more meioses become informative. The polymorphism information content (PIC) is a numerical index of the usefulness of a given marker (Botstein et al, 1980).

2.1.3 Ideal Family Structure for Linkage Studies on the X Chromosome

In X-linked diseases recombinations only occur in female meioses. Therefore, if a male in generation I has a carrier daughter in generation II all meioses in that female will be phase known, providing paternity is certain and the disorder is inherited and does not represent a new mutation. As carrier status of females is often not certain on clinical grounds it is more useful to collect samples from males in generation III. An additional advantage of using only male offspring in generation III is that non-paternity does not affect interpretation of results. Hence, the ideal family structure is a three generation pedigree with a male in generation I who has a daughter in generation II with male offspring in generation III. Families with rare X-linked disorders, however, often do not fulfill these criteria.

2.1.4 Analysis of Pedigree Data for Linkage Studies in Man

Linkage data for two and three generation families is analysed using the lod score method devised by Newton E. Morton (Morton, 1955). The lod score is the logarithm to the base 10 of the likelihood ratio, or odds, for or against linkage. The likelihood ratio is the ratio of the likelihood of the pedigree data given linkage at a certain recombination fraction, θ , compared to the likelihood of the same pedigree data given no linkage (recombination fraction, θ , of 0.5). Lod scores are calculated for recombination fractions from 0 to 0.45. The position at which the highest lod score is found is the maximum likelihood estimate of the interval separating two loci. Since lod scores are logarithms, information from separate families can simply be added. A total lod score of +3.0 is generally taken as proof of linkage at a given value of θ and a total lod score of -2.0 is generally taken as exclusion of linkage. When the prior probability of 50:1 that two random markers will not be linked are taken into consideration a lod score of +3.0 means that the odds in favour of linkage are 20:1. However if two loci are already known to be on the same chromosome the a priori odds against linkage are lower and therefore a lower lod score will give the same odds in favour of linkage. A lod score of +2.0 is often accepted as indicating linkage if both loci are already known to lie on the X chromosome. Using these criteria about one in twenty independent linkages will be spurious although this is less likely with multilocus analysis.

Lod scores between two loci can be calculated using the computer programme LIPED (Ott, 1974). Multilocus linkage analysis can be performed using the LINKAGE programme which allows simultaneous use, or integration, of information from multiple loci in a segment of a chromosome and is therefore more efficient (Lathrop et al, 1984).

2.2 Chromosome Rearrangements

2.2.1 X;autosome Translocations

In females carrying X;autosome translocations the normal X chromosome is inactive in all cells. The female is therefore effectively hemizygous and if the translocation disrupts a gene on the X chromosome she will manifest the same signs as a male with a mutation in that gene. Observations of X;autosome translocations have been used to localise the genes which are defective in Duchenne muscular dystrophy (Greenstein et al, 1977), Hunter's syndrome (Mossman et al, 1983) and X-linked ectodermal dysplasia (Gerald and Brown, 1974).

2.2.2 Deletion Mapping

Males who have more than one X-linked disorder, or mental retardation and an X-linked disorder not usually associated with retardation, may have a deletion on the X chromosome. A male with Duchenne muscular dystrophy, chronic granulomatous disease, retinitis pigmentosa and McLeod syndrome and an interstitial deletion of Xp21 localised these conditions to this band (Francke et al, 1985). Another male with mental retardation, Duchenne muscular dystrophy, adrenal hypoplasia and glycerol kinase deficiency was found to have a deletion in Xp21 mapping these two further conditions to the same area (Renier et al, 1983).

In addition to providing gene mapping information, males with X chromosome deletions provide a way of cloning DNA sequences from these regions. When DNA containing normal X chromosomes is digested with an endonuclease and then reassociated with an excess of sheared DNA from the individual with the deletion the only fragments that can be cloned into a vector cut with the same endonuclease map to the deleted region. This was done with the first deletion above, using phenol (Kunkel et al, 1985) and high inorganic phosphate (Smith et al, 1987) to enhance the reassociation.

2.3 Conserved Linkage Groups

Ohno's law states that the X chromosomes of all mammals carry homologous genes. The chromosome rearrangements that have occurred in the X chromosome of mouse and man have been studied in detail (Amar et al, 1988) and, therefore, when there is a mouse model of a human X-linked disorder localisation of the mouse mutation allows predictions about the corresponding human disease locus.

2.4 Somatic Cell Hybrids

Somatic cell hybrids are made by fusing cells from a human fibroblast or lymphoblastoid line with cells from a rodent line. The two cell lines are grown in culture together and treated with polyethylene glycol or Sendai virus to induce fusion. Some of the heterokaryons produced go on to form hybrid cells with nuclei containing both human and rodent chromosomes. The hybrid cells preferentially lose human chromosomes. Each clonal population of cells is picked, cultured separately and karyotyped. The probe is hybridised to Southern blots of restriction endonuclease digested hybrid DNA. Localisations are made by constructing a table showing which chromosomes were present in hybrids which gave a signal and which chromosomes in hybrids that did not give a signal hence excluding localisation of the probe from certain chromosomes leaving a small group of possible chromosome assignments.

It is possible to select for hybrids containing the X chromosome by fusing the human cells with a rodent line deficient for hypoxanthine-guanine ribosyl transferase (HPRT). The hybrid is then grown in HAT (hypoxanthine, aminopterin, thymidine) medium. Aminopterin is a folic acid antagonist and will inhibit purine and pyrimidine metabolism. The pyrimidine pathway can be salvaged in the presence of thymidine by thymidine kinase and the purine pathway can be salvaged by the conversion of hypoxanthine to inosine 5'-monophosphate by HPRT which maps to Xq26. It is also possible to select against hybrid lines expressing HPRT by adding the purine analogues, 6-thioguanine or 8-azaguanine, to the culture medium. Normal cells incorporate these but HPRT⁻ cells do not incorporate them and therefore survive.

In the case of loci on the X chromosome localisation to the X is usually obvious from the inheritance within families but somatic cell hybrids can be very useful in providing regional assignments using cell hybrids containing X;autosome translocations. The smallest region of overlap which gives a positive hybridisation signal from a panel of hybrids containing different translocations allows regional localisation. Hybrids containing small fragments of human chromosomes can also be produced by irradiating the human cells before the formation of the hybrid. Accuracy of results from somatic cell hybrid work depend on detailed cytogenetic analysis.

2.5 In Situ Hybridisation

In situ hybridisation is a direct method of mapping. Nucleic acid sequences are hybridised to their complementary DNA in metaphase chromosome spreads fixed on glass slides. Using in situ hybridisation a probe can be localised to within a single band of a 300 band karyotype (approximately 10 Mb). The probe DNA can either be labelled

using a radioactive isotope, usually ^3H , detected by autoradiography, or using non-radioactive techniques such as biotinylated probes. The method was first used to localise repetitive DNA (Gall and Pardue, 1969) but as improvements have occurred single copy sequences have been localised. Recently sequences of 1 kb have been mapped using biotinylated probes (Garson et al, 1987).

2.6 Pulsed Field Gel Electrophoresis

The methods of mapping described above have a maximum resolution of several megabases. Having achieved this level of localisation pulsed field gel electrophoresis can be used for finer mapping as this allows separation of DNA fragments from 20 kb up to 9 Mb (Schwartz and Cantor, 1984; Barlow and Lehrach, 1987). The method uses two alternating electric fields at an obtuse angle to each other. When molecules are subjected to an electric field at an angle to their field of migration they reorientate themselves along the new field before moving forwards, small molecules reorientate faster than large molecules and so have a higher mobility. The DNA tracks on the gels produced by orthogonal field alternation gel electrophoresis (OFAGE), the first method of pulsed field gel electrophoresis, curve away from the midline making it difficult to estimate band sizes accurately. One of the modifications made to give straighter tracks uses a hexagonal electrode configuration to generate a uniform electric field at all points on the gel apparatus (Chu et al, 1986). This electrode configuration is referred to as CHEF, which stands for clamped homogeneous electric fields. The fragment sizes separated and the region of maximum resolution can be adjusted by altering the switch interval, voltage and length of run. The DNA is transferred from the gel onto nylon filters and hybridised with the probes using standard techniques. Restriction fragment maps can be generated around each probe until they overlap giving a map of the region.

When separating DNA fragments in this size range it is important that the DNA isolated from cells is of very high molecular weight. DNA made by standard methods is subject to shearing and is not adequate for pulsed field gel electrophoresis. Methods have been developed of isolating DNA from cells suspended in agarose blocks to minimise degradation (Schwartz and Cantor, 1984). Enzymes have to be chosen which generate DNA fragments in the size range being separated. Enzymes that cut genomic DNA rarely either have a long recognition sequence or CpG in their recognition sequence. Sfi has been one of the most useful endonucleases for pulse field mapping and its recognition sequence is GGCCNNNGGCC. Most of the other enzymes used are methylation sensitive endonucleases with a CpG in the recognition sequence, for example the NotI recognition sequence is GCGGCCGC and the BssHII recognition sequence is GCGCGC.

The dinucleotide, CpG, is found at a fifth of the expected frequency from the base composition of genomic DNA, which is AT rich. In addition 60-90% of CpG dinucleotides are methylated. However there are short sections of DNA where CpG dinucleotides occur at the predicted frequency and are not methylated. These regions of DNA are referred to as HTF islands because HpaII, with its recognition sequence CCGG, digests DNA from these regions to give tiny fragments (HpaII Tiny Fragments) (Bird et al, 1985). These islands are always associated with genes although not all genes have HTF islands (Gardiner-Garden and Frommer, 1987). Many of the endonucleases used to generate restriction maps with pulse field gel electrophoresis cut within HTF islands. 89% of NotI sites are in HTF islands and 74% of BssHII sites are within HTF islands (Lindsay and Bird, 1987). A cluster of digestion sites using endonucleases which recognise unmethylated CpGs implies an HTF island and hence the position of a gene. Therefore pulsed field gel electrophoresis can be used to generate a map of a region and also pinpoint the site of genes with HTF islands in that map.

2.7 Mapping Studies of Proximal Xq

Detailed mapping studies provide information of immediate clinical relevance. The more probes, detecting restriction fragment length polymorphisms, that are mapped to the area of a disease locus the greater the probability of a woman being heterozygous for a linked probe. Hence, predictive tests can be offered to more families. In addition, probe order is essential information for refining the localisation of the disease and for building up a physical map of the region which is a preliminary step in cloning the defective gene.

Following the localisation of X linked agammaglobulinaemia to Xq21.3-22 and X-linked severe combined immunodeficiency to Xq11-13 a study was started to determine the order of DNA probes known to be between the centromere and Xq22. A panel of X-linked probes was tested in reference families that had been noted to have a recombination on Xq and in a male with a deletion on Xq to give probe ordering data. The same probes were then used to refine the mapping information about XSCID and XLA. In addition physical mapping of the probes in Xq21.3-22 was started using pulsed field gel electrophoresis.

CHAPTER 3. Introduction: Use of X Inactivation Studies in the Investigation of Severe Combined Immunodeficiency

Severe combined immunodeficiency is a group of disorders that can be inherited in X-linked or autosomal recessive forms. All the children have absent T cell function but there is considerable variation in T and B cell numbers and it is not possible to distinguish between autosomal and X-linked forms on the basis of immunological tests. Female carriers of XSCID do not have any known biochemical or immunological abnormalities.

In families where there is a clear X-linked pedigree carrier detection can be offered using restriction fragment length polymorphisms detected by probes that are linked to the disease. However most of the families referred to The Hospitals for Sick Children have only one affected male child and no previous family history, or a history of males in previous generations on the maternal side dying in infancy but not having any further information. It was therefore important to develop a method of clarifying the inheritance pattern in these families so that more accurate genetic counselling could be given to the couple. In addition knowing the inheritance pattern would affect the management of the extended family: in X-linked families female relatives should be investigated but in autosomal recessive cases relatives could be reassured that the risk of having a child with the same disease is extremely low.

3.1 X Inactivation

The Lyon hypothesis states that permanent inactivation of one of the two X chromosomes occurs at random in every somatic cell in the female early in embryogenesis. The pattern of X chromosome inactivation is transmitted in stable fashion to all progeny cells. In a mature cell population therefore approximately half of the cells will have the paternal X active and the remaining cells will have the maternal X active.

3.1.1 X Chromosome Usage in Females carrying X-linked Disorders

In female carriers of some X-linked disorders there are two populations of cells, one normal and one abnormal, as expected from the Lyon hypothesis. Female carriers of chronic granulomatous disease have normal and abnormal granulocytes when phagocytic function is tested with nitrotochrome blue. Some cultured fibroblast clones in female carriers of Hunter's syndrome accumulate polysaccharide, and presumably have the abnormal X active, and others are normal.

In three X-linked conditions a non random population of active X chromosomes has been demonstrated in mature cell populations by investigating women who were also heterozygous for glucose-6-phosphate

dehydrogenase (G6PD) isoenzymes. The first condition in which non-random X chromosome usage was found was the metabolic disorder Lesch Nyhan syndrome, which results from hypoxanthine-guanine-phosphoribosyl transferase (HPRT) deficiency.

The other two conditions, Wiskott-Aldrich syndrome and Bruton's agammaglobulinaemia, are both disorders of the immune system.

Assays of HPRT activity in obligate carriers of Lesch-Nyhan syndrome showed erythrocyte levels to be in the normal range. Nyhan investigated two obligate carriers of the disease in one family who were heterozygous for G6PD types A and B (Nyhan et al, 1970). Erythrocytes and lymphocytes expressed only type B G6PD, fibroblasts expressed both type A and type B. These results suggested that the same X chromosome was active in all erythrocytes and lymphocytes and that the X chromosome carrying the abnormal HPRT gene was inactive in these cells.

An obligate female carrier of Wiskott-Aldrich syndrome who was heterozygous for G6PD types A and B has been investigated (Gealy et al, 1980). Granulocytes, platelets, monocytes, B lymphocytes and T lymphocytes had only the A isoenzyme detectable. The ratio of A to B in her erythrocytes was 3:1 and in cultured skin fibroblasts the isoenzymes A and B were expressed in equal proportions. Again this suggested that in some mature cell populations all the cells had the same X chromosome active.

An obligate carrier of Bruton's agammaglobulinaemia heterozygous for G6PD isoenzymes A⁻ and B has been investigated. Neutrophils and mononuclear cells expressed both isoenzymes but only the B isoenzyme was detected in B cells (Conley et al, 1986).

It can be concluded from these four examples that although the initial X inactivation process is random a non random pattern of X inactivation can be found in selected mature cell populations of women carrying some X-linked disorders.

3.1.2 Methods of Investigating X Chromosome Usage

The simplest method of studying X inactivation patterns is to use glucose-6-phosphate dehydrogenase polymorphisms detected by starch gel electrophoresis of cell lysates. This method is rapid, simple and requires relatively few cells. However very few caucasian women are heterozygous for the polymorphic variants of this protein and therefore it would not be useful as a routine test in our population.

Another method for studying X inactivation patterns is to make somatic cell hybrids and select for hybrids containing the active X chromosome by culturing in HAT medium. Hybrid clones that retain two human X chromosomes can be grown in medium containing 6-thioguanine which is toxic to cells expressing HPRT and, hence, any cells surviving in this medium will have lost the active X. One can then use

any polymorphic X-linked trait that the woman is heterozygous for to test whether X chromosome use is random or not (Puck et al, 1987). Whilst this method has the advantage that nearly all women will be heterozygous for one of the protein or DNA polymorphisms on the X chromosome it obviously takes considerable time and resources for each woman investigated.

Recently a method has been developed for investigating X inactivation patterns based on methylation differences between the active and inactive X chromosome (Vogelstein et al, 1987). Probes from the 5' end of the HPRT and the phosphoglycerate kinase (PGK) genes detect both a polymorphism and a difference in methylation between the active and the inactive X chromosome. One endonuclease detects the polymorphism and then methylation-sensitive endonucleases (HpaII and HhaI) are used to demonstrate the methylation pattern.

3.1.3 The PGK Locus

The PGK probe, pSPT/PGK, detects a BstXI polymorphism and a BglII polymorphism. These polymorphic sites are in linkage disequilibrium.

A restriction map of the 5' end of the PGK gene is shown in figure 3.1. The polymorphic sites and HpaII sites are marked on the figure. The recognition site of HpaII is CCGG but HpaII only digests DNA when the CpG dinucleotide is unmethylated. The first eight HpaII sites are methylated on the inactive X chromosome and unmethylated on the active X chromosome (Keith et al, 1986). The ninth HpaII site is unmethylated on the active X chromosome but is also unmethylated on 20-40% of inactive X chromosomes.

Two combinations of restriction endonucleases were used to look at X inactivation patterns with this probe. In the first method BglII and EcoRI were used to bracket the BglI polymorphic site, after digestion with these three endonucleases the sample was divided and HpaII added to one aliquot. Polymorphic bands of 1.7 and 1.3 kb, which are very clear on autoradiographs, are generated using this combination of endonucleases. In a non random population the band representing the active X chromosome will become much smaller, the band representing the inactive X chromosome will remain in the same position but will be fainter due to digestion at the ninth HpaII site.

In the second combination of enzymes BstXI is used to detect the polymorphism and PstI added to exclude the ninth HpaII site. The polymorphic bands generated are 1.05 and 0.9 kb. This method avoids the difficulties raised by the variable methylation of the ninth HpaII site but has the disadvantage that the bands on the autoradiograph are less sharp.

Both combinations of endonucleases were used at different times during the investigations.

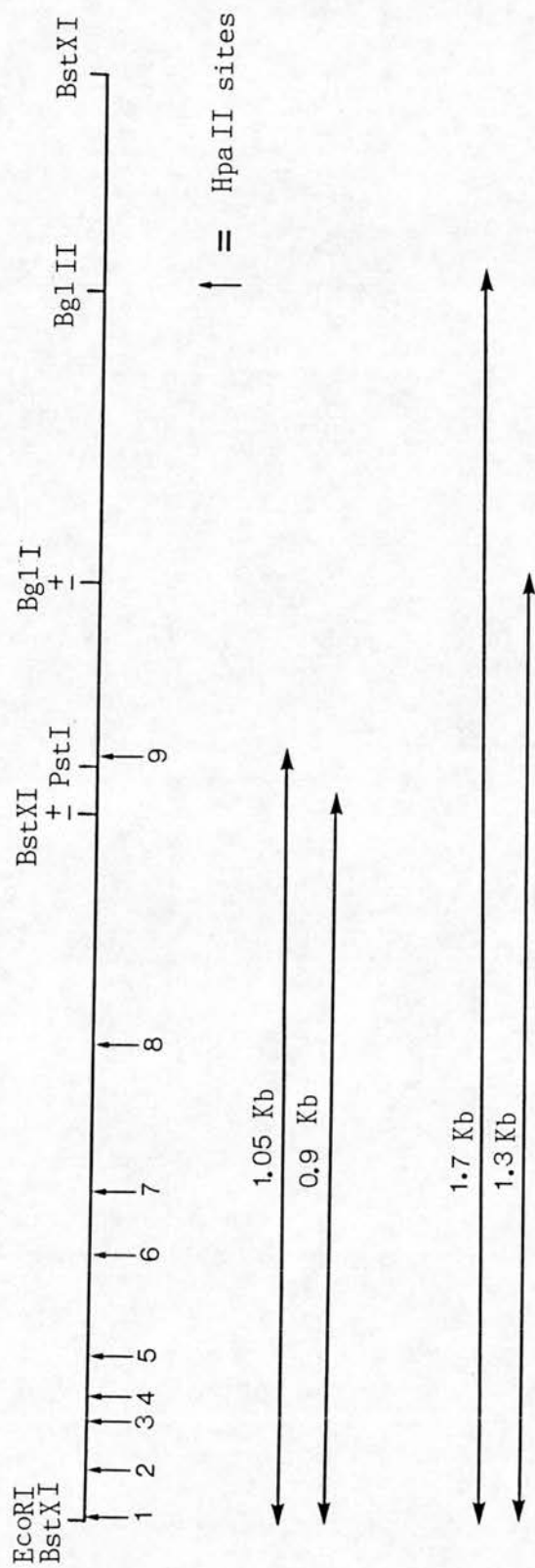


Figure 3.1 A restriction fragment map of the 5' end of the PGK gene. The fragments resulting from BstXI/PstI digestion and BglI/BglII/EcoRI digestion are also shown.

3.1.4 The HPRT Locus

A BamHI polymorphism of 24/12kb is detected by two contiguous probes taken from the 5' end of the HPRT gene. Addition of PvuII is recommended as this reduces the band sizes by 6kb to 18/12kb improving transfer of the DNA during blotting and allowing the test to be carried out even if the DNA is slightly degraded (figure 3.2).

HhaI recognizes the sequence CGCG but only digests the DNA when the CpG dinucleotides are unmethylated. There are 6 HhaI sites within the DNA fragment that is detected by the probes after BamHI/PvuII digestion. The first HhaI site is unmethylated on most active X chromosomes and also on some inactive X chromosomes. HhaI sites 2-6 are methylated in over 95% of active X chromosomes but at least one of these sites is unmethylated in over 90% of inactive X chromosomes.

Hha I Sites	Active X	Inactive X
Site 1	most unmethylated	some unmethylated
Site 2-6	methylated >95%	1 unmethylated >90%

Table 3.1 Methylation pattern at the HhaI restriction sites in the BamHI DNA fragment detected by HPRT-600 and HPRT-800

In more than 95% of active X chromosomes the DNA fragment will become only 200bp smaller after the addition of HhaI as the DNA will only be cut at site 1. The DNA fragment will be 10kb or less for more than 90% of inactive X chromosomes after the addition of HhaI, depending on which HhaI sites are unmethylated (figure 3.3).

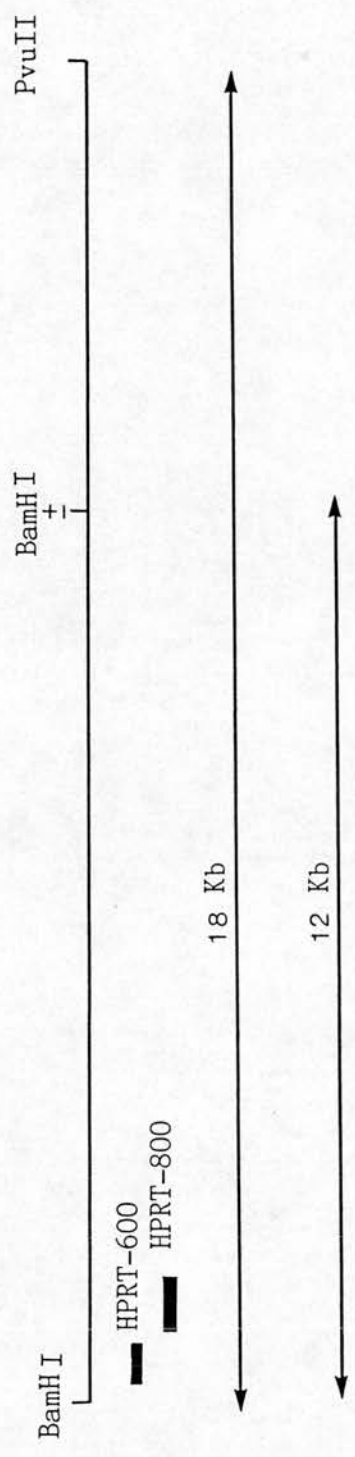


Figure 3.2 Restriction map of the 5' end of the HPRT gene showing the HPRT-600 and HPRT-800 probes and the fragment sizes that they detect after BamHI/PvuII digestion.

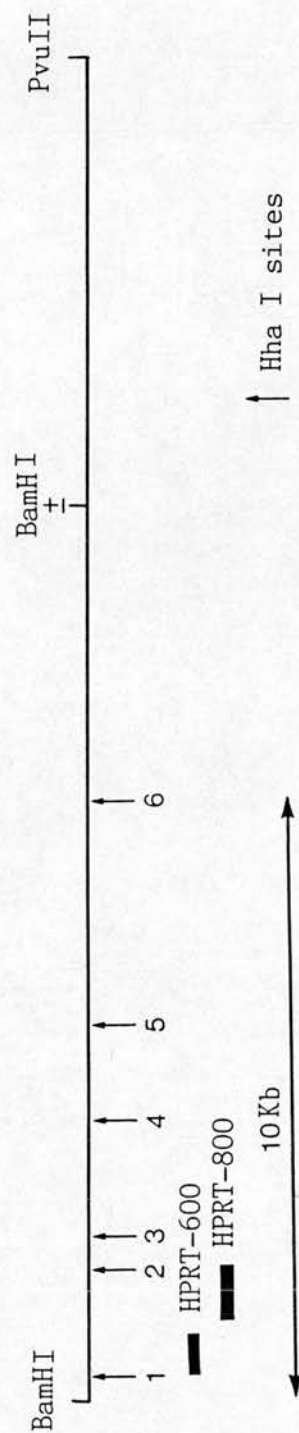


Figure 3.3 Restriction map of the HPRT gene demonstrating that when one of sites 2-6 is unmethylated the fragment detected by the probes is less than 10kb.

HpaII recognizes the sequence CCGG but only digests DNA when the CpG dinucleotide is unmethylated. There are 9 HpaII sites within the Bam/PvuII fragment detected by the HPRT probes.

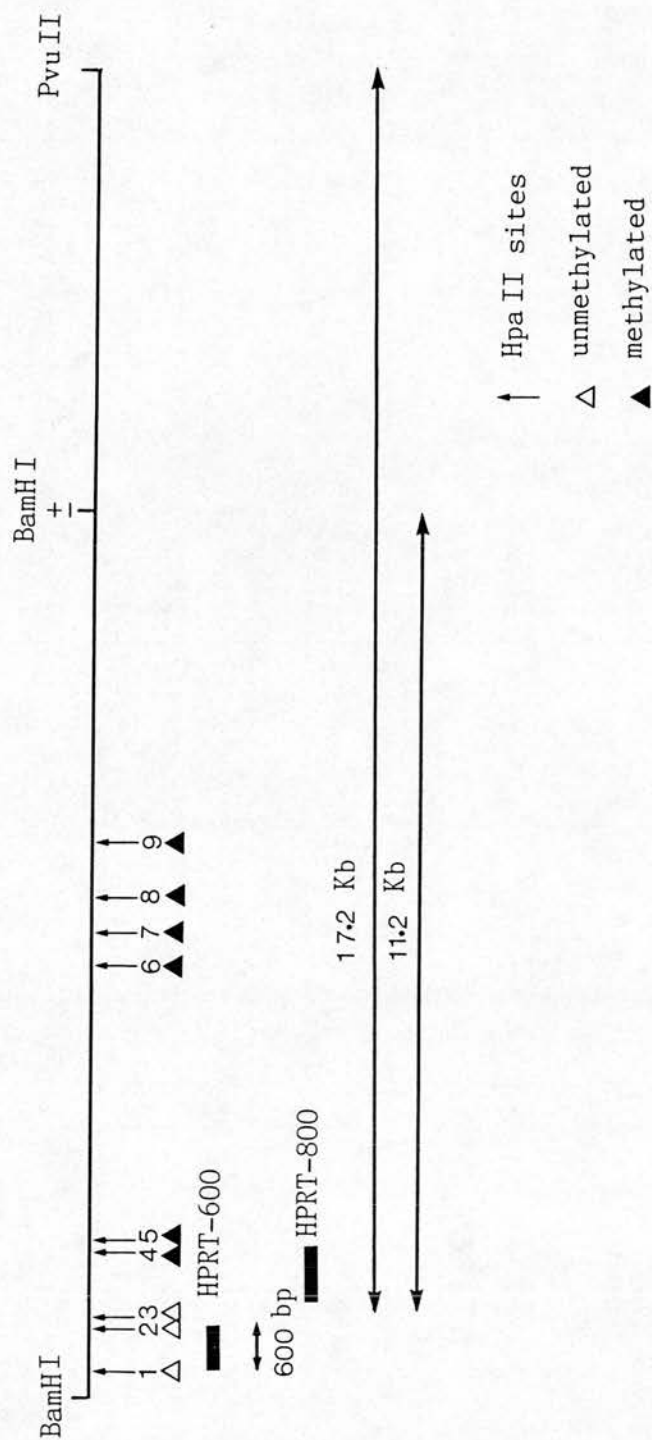
All active X chromosomes have the same pattern of methylation at the HpaII sites but the methylation pattern at these sites on inactive X chromosomes differs even within the same subject. The first HpaII site is unmethylated on active X chromosomes and also on some inactive X chromosomes. Sites 2 and 3 are unmethylated on the active X and methylated on the inactive X. Sites 4-9 are methylated on the active X and on type II inactive X chromosomes, but at least one is unmethylated on type I inactive X chromosomes. Most women have a mixture of Type I and type II inactive X chromosomes with type I accounting for 60-100% of inactive X chromosomes.

HpaII Sites	Active X	Inactive X	
		Type I	Type II
Site 1	unmethylated	unmethylated in some	
Sites 2 and 3	unmethylated	methylated	methylated
Sites 4-9	methylated	at least 1 unmeth	methylated

Table 3.2 Methylation pattern at the HpaII restriction sites in the BamHI/PvuII fragment detected by the HPRT-600 and HPRT-800 probes

If a filter of DNAs digested with BamHI/PvuII and HpaII is probed with the HPRT-800 probe then the active X appears 800bp smaller, type I inactive X chromosomes are less than 8kb but the type II inactive X chromosomes remain at the original size. These autoradiographs are difficult to interpret because over the distances that the gels are run 17.2 kb and 18 kb cannot be clearly distinguished. If the same filter is hybridized with the HPRT-600 probe the band representing the active X is much smaller at 600bp while the bands for the type I inactive X chromosomes are between 600bp and 8kb and the type II inactive X chromosomes remain in the original position. In any woman who has a non random X inactivation pattern it will be seen clearly on this filter if she has type II inactive X chromosomes but in those women where nearly all inactive X chromosomes are type I both the bands will be lost and interpretation will not be possible.

The bands detected by HPRT-600 and HPRT-800 after BamHI/PvuII/HpaII digestion of active X chromosomes are shown in figure 3.4 and the bands detected after BamHI/PvuII/HpaII digestion of type I and type II inactive chromsomes are shown in figure 3.5.



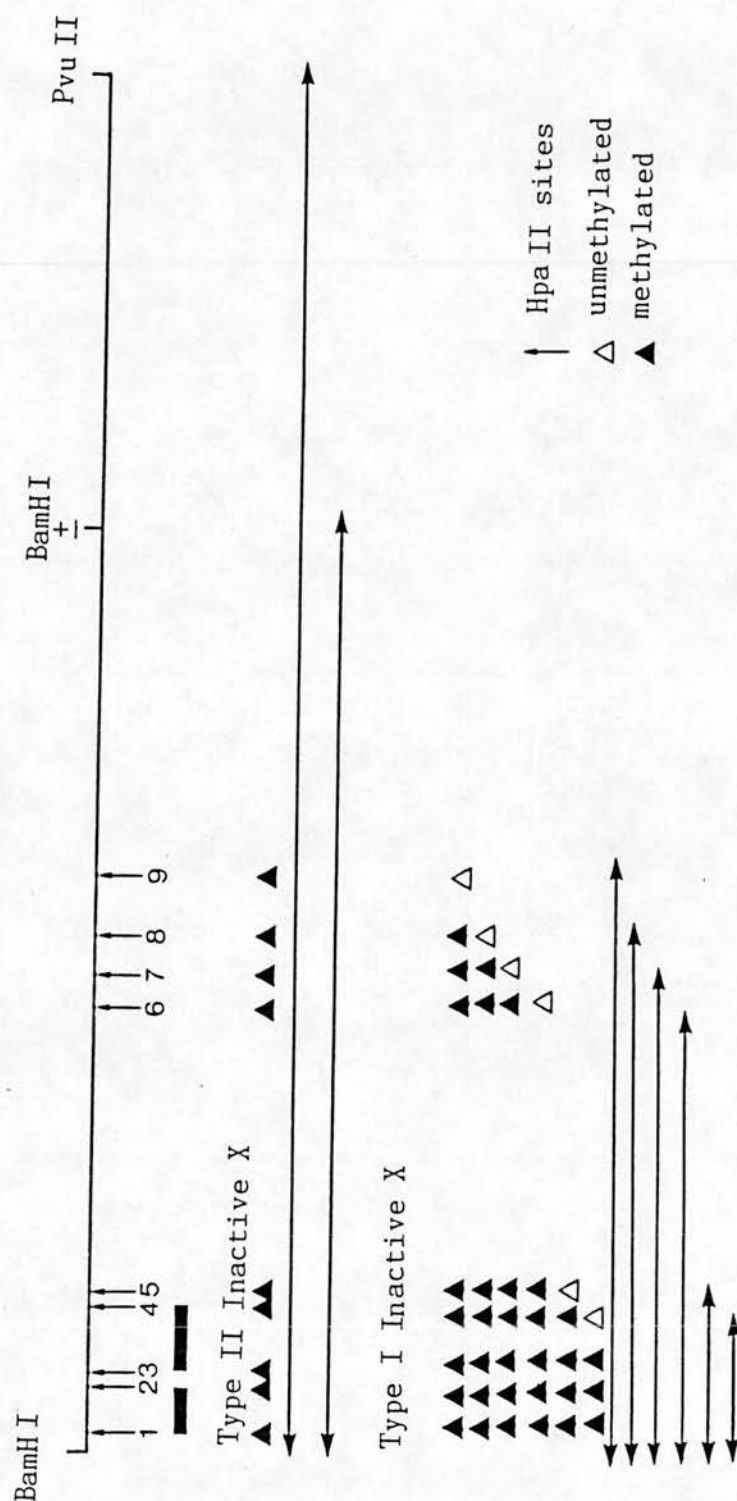


Figure 35 The methylation pattern at HpaII sites in Type I and Type II Inactive X Chromosomes and the fragment sizes detected by the HPRT-600 and HPRT-800 probes after digestion with BamHI/PvuII/HpaII

3.2 Hypothesis

The observations in XLA and Wiskott-Aldrich syndrome led to the hypothesis that as males with X-linked severe combined immunodeficiency have abnormal T cell development female carriers of the disease would have a non-random pattern of X inactivation in their T cells and this could provide the basis for a carrier test. Vogelstein's data suggested that approximately fifty percent of caucasian women are heterozygous for the polymorphisms detected by HPRT or PGK. These probes were used to investigate families referred to The Hospitals for Sick Children and to investigate the expression of the gene defective in XSCID in other haematopoietic cells.

3.2.1 Investigation of Consultands

Initially a 10 mls sample of blood was taken from each female referred for investigation. DNA from this sample was tested to see if she was heterozygous for either the PGK or the HPRT polymorphisms. Women who were heterozygous for either of the polymorphisms were then asked to attend the clinic where the study was explained to them. 50 mls of blood was taken from the women who wished to participate for cell separation and methylation analysis.

CHAPTER 4. Methods

4.1.1 Phenol/chloroform extraction of DNA

An equal volume of phenol saturated with 50mM Tris-HCl pH8.0 was added to the solution containing the DNA and, after thorough mixing, the sample centrifuged. The upper aqueous layer containing the DNA was gently removed using a wide mouth glass pipette and a second extraction performed using $1/2$ volume phenol saturated with 50mM Tris-HCl pH8.0 and $1/2$ volume chloroform. The upper aqueous layer was again removed and extracted twice using an equal volume of chloroform to remove residual traces of phenol.

4.1.2 Ethanol precipitation of DNA

DNA was precipitated by adding $1/10$ volume 3M sodium acetate pH5.2 and $2 1/2$ volumes ice-cold ethanol or by adding $1/2$ 7.5M ammonium acetate followed by 2 volumes ice-cold ethanol.

4.2 DNA Extraction

4.2.1 DNA Extraction from Blood Samples

Blood samples were collected into tubes containing the anticoagulant ethylenediamine tetraacetate (EDTA) and stored at -70°C . The following protocols give the volumes of reagents used for 10mls of blood. Method 1 was used at the beginning of the study but after publication of the second method it was used in preference because it reduced the time taken for DNA extraction and also avoided the use of phenol with its associated hazards.

Method 1

The frozen blood was thawed at room temperature. 40 mls of ice-cold distilled water was added to lyse the red cells. The sample was centrifuged for 20 minutes at 800g at 4°C . The supernatant was decanted and the white cell pellet disrupted by the addition of 25 mls of ice-cold 0.1% NP40. After mixing the sample was centrifuged for a further 20 minutes at 800g at 4°C . The supernatant containing the cell debris was decanted and the nuclear pellet was resuspended in 2.5mls of 75mM NaCl/24mM EDTA pH8.0. When the pellet was completely dispersed 125 μl of 10% sodium dodecyl sulphate (SDS) and 1mg of proteinase K were added and the sample incubated at 37°C overnight or at 56°C for two hours and then 2.5mls of 75mM NaCl/24mM EDTA pH8.0 added. Phenol chloroform extractions were then carried out as described in section 4.1.1, spinning the sample at 1800g for 20 minutes for the first two extractions and then at 1800g for 10 minutes for the chloroform extractions. After ethanol precipitation the DNA pellet was removed using a glass pipette with a sealed tip and air dried for 5 minutes before dissolving in 0.5mls TE. The sample was then placed on a rotary mixer in a room at 4°C for 48 hours prior to storing at -20°C .

Method 2

The nuclear pellet was prepared as in method 1 but then resuspended in 14mls 6M guanidine hydrochloride and 1ml 7.5M ammonium acetate. 1ml 20% sodium sarkosyl and 1.5mg proteinase K were added and the solution incubated at 60°C for 90 minutes. The DNA was then precipitated by the addition of 35mls of ethanol and the DNA pellet spooled out using a sealed glass pipette. The sample was dissolved in 0.5mls TE overnight and then reprecipitated using 3M sodium acetate and ethanol. The DNA pellet was spooled out and redissolved in TE.

4.2.2 DNA extraction from cells

DNA was extracted from cells using the above methods but omitting the step for lysis of red cells. The above protocols give the volumes suitable for approximately 50×10^6 cells and were scaled down accordingly.

4.2.3 DNA extraction from chorionic villus

The sample was examined under a low-power dissecting microscope and any contaminating maternal tissue removed. The sample was washed once in 75mM NaCl/24mM EDTA pH8.0 in an eppendorf tube. 0.5mls of NaCl/EDTA, 25µl 10% SDS and 0.2mg proteinase K were added and the sample incubated at 56°C for 1 - 2 hours. Phenol chloroform extractions were carried out as in section 4.1 spinning the sample for 4 minutes in a microfuge at each stage. The DNA was then ethanol precipitated, spooled out and dissolved in 50µl TE.

4.2.4 DNA extraction from placenta for pre-annealing

Placental tissue was cut into small pieces and homogenised in 75mM NaCl/24mM EDTA in a tissue homogeniser and the homogenate sieved through muslin. 1 mg proteinase K was added per gram of placenta and SDS to a concentration of 0.5%. After overnight incubation at 37°C phenol chloroform extractions and ethanol precipitation were carried out as in sections 4.1 and 4.2. The DNA was sonicated to 500-100bp and kept at a concentration of 10 mg/ml

4.2.5 Measurement DNA Concentration

A 15µl aliquot was made up to 750µl with distilled water and the optical density read at 260nm and 280nm. As an optical density(OD) of 1 at 260nm corresponds to 50µg/ml double stranded DNA multiplying the reading by 2.5 gave the concentration in mg/ml when using this dilution. The purity of the sample was assessed by the ratio OD_{260}/OD_{280} . A pure sample gives a ratio of 1.8 and a sample contaminated by phenol or protein gives a lower ratio.

4.3 Restriction Enzyme Digestion

4.3.1 Standard Restriction Enzyme Digest

5µg of DNA was digested with 10 units of enzyme for 3 hours in a volume of 40µl using the buffer and temperature conditions recommended by the manufacturer but with the addition of 3mM spermidine trihydrochloride. Any sample that failed to digest was precipitated using ammonium acetate and ethanol prior to redigestion. 4µl of loading buffer (50% glycerol, 1mM EDTA, 0.25% bromophenol blue) was added to each sample.

4.3.2 Restriction Enzyme Digest for Methylation Analysis using HPRT

15µg DNA was digested with 40 units of PvuII and 40 units of BamHI in 100mM NaCl, 10mM MgCl₂, 10mM Tris-HCl pH8.0, 3mM spermidine and 100µg/ml bovine serum albumin (BSA) in a reaction volume of 80µl. The DNA was then ammonium acetate/ethanol precipitated with 20µg glycogen present as a carrier. After 1 hour at -20°C the sample was centrifuged in a microfuge for 30 minutes. The DNA pellet was dried in vacuo, resuspended in 90µl of TE and divided into three equal aliquots. One aliquot was digested with 10 units of HhaI in 50mM Tris-HCl pH8.0, 5mM MgCl₂, 6mM mercaptoethanol and 100µg/ml bovine serum albumin. Another aliquot was digested with 10 units of HpaII in 6mM KCl, 10mM Tris-HCl pH7.4, 10mM MgCl₂, 10mM mercaptoethanol, 100µg/ml BSA and 3mM spermidine. The three samples were loaded in adjacent wells so that the BamHI/PvuII digest would demonstrate the position of the polymorphic bands and act as an intensity control as the three lanes contained an equal amount of DNA.

4.3.3 Restriction Enzyme Digest for Methylation Analysis using PGK

1) 15µg DNA was digested with 30 units of PstI in 50mM Tris-HCl pH8.0, 10mM MgCl₂, 50mM NaCl, 100µg/ml BSA and 3mM spermidine for 3 hours at 37°C in a reaction volume of 80µl, 30 units of BstXI was added and the sample incubated for a further 3 hours at 56°C.

The DNA was ammonium acetate/ethanol precipitated with 20µg glycogen present as a carrier. After 1 hour at -20°C the sample was centrifuged in a microfuge for 30 minutes. The DNA pellet was dried in vacuo and then resuspended in 60µl TE. This was divided into two equal portions. One portion was digested with 10 units of HpaII for 3 hours at 37°C in 6mM KCl, 10mM Tris-HCl pH7.4, 10mM MgCl₂, 10mM mercaptoethanol, 100µg/ml BSA and 3mM spermidine. The two samples were loaded in adjacent wells so that the PstI/BstXI digest showed the position of the polymorphic bands and acted as a dosage control.

2) 15µg DNA was digested with 30 units of BglI, 30 units of BglII and 30 units of EcoRI in 50mM Tris-HCl pH8, 10mM MgCl₂, 50mM NaCl, 10mM mercaptoethanol, 100µg/ml BSA and 3mM spermidine at 37°C. The DNA was ammonium acetate/ethanol precipitated as in part 1 and divided into

two equal portions, one to be digested with HpaII and one for the control track.

4.4 Southern Blotting

4.4.1 Preparation of the Gel, Electrophoresis and Transfer of DNA

An 0.8% agarose gel was prepared by dissolving agarose in electrophoresis buffer by boiling, cooling the solution to 50°C in a water bath and then adding ethidium bromide to a final concentration of 0.5µg/ml immediately prior to pouring the gel. When set the gel was placed in the electrophoresis tank and electrophoresis buffer (40mM Tris, 20mM sodium acetate, 2mM EDTA, the pH of the 10 X stock solution was adjusted to 7.7 using glacial acetic) added to cover the gel to a depth of about 1mm. Samples were loaded into the wells leaving one blank track for orientation of autoradiographs and with HindIII digested lambda or BstEII digested lambda as a size marker in one track. Electrophoresis was carried out at 1.5V/cm for 16 hours to detect most polymorphisms. After electrophoresis the gel was placed next to a ruler on an ultraviolet transilluminator and photographed. The gel was denatured for 1 hour in 0.5M NaOH, 1.5M NaCl and then neutralised in 0.5M Tris-HCl pH8.0, 1.5M NaCl for 1 hour changing the neutralising solution once. The Southern blot was then set up for transfer of DNA onto a nylon filter (Hybond N, Amersham) as shown in figure 4.1. After overnight transfer the position of the wells and the number of the filter were marked on the filter with a permanent marker. The filter was rinsed in 2 X SSC (appendix 2) and baked for 2 hours at 80°C. Filters were stored at room temperature until hybridised.

4.4.2 Prehybridisation and Hybridisation of Filters

The filters were rinsed in 2 X SSC and placed in polythene bags with a maximum of three filters per bag. 7 mls of hybridisation solution (appendix 2) was added to the bag which was then sealed and incubated at 65°C for three hours. The filters were then transferred to hybridisation solution containing 10⁶dpm/ml denatured labelled probe and incubated at 65°C overnight.

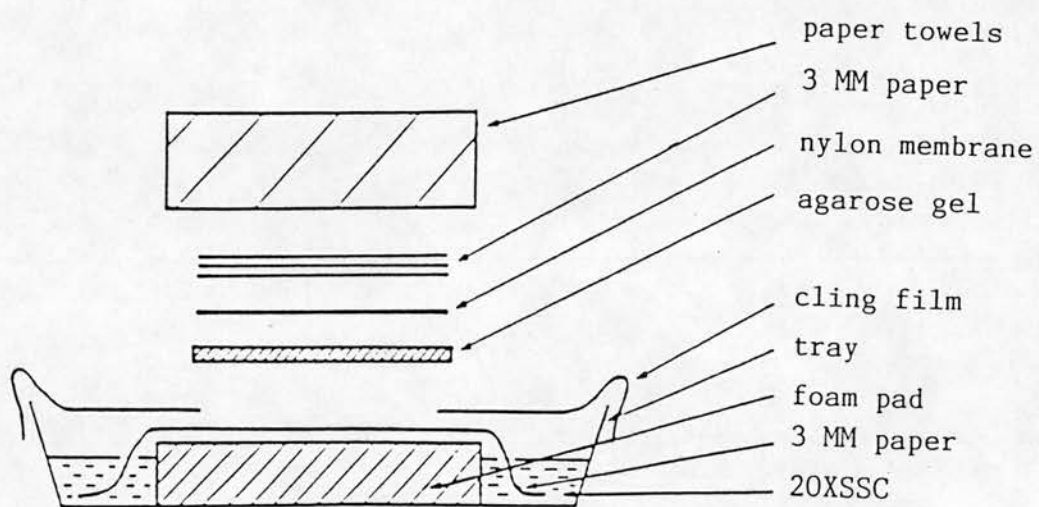


Figure 4.1 Method of transfer of DNA from agarose gel to nylon filter

4.4.3 Washing Filters

Filters were removed from the hybridisation bags and washed in 3 X SSC, 0.1% SDS at room temperature three times for twenty minutes and then monitored with the beta-counter. The temperature was increased to 65°C and the filters washed at increasing stringency changing the wash solution at thirty minute intervals and monitoring with the beta counter at each change until the counts were acceptable. When the wash solution was changed the SSC concentration was progressively decreased to 1 X SSC, 0.5 X SSC, 0.2 X SSC, and if necessary 0.1 X SSC. The washed filters were wrapped in clingfilm, the position of the wells and the number of the filter marked with a fluorescent pen (Ultemit, Dupont), and exposed to Kodak XAR-5 film in cassettes with intensifying screens at -70°C. The length of exposure required was dependent on the probe and varied from 3 hours to 10 days.

4.4.4 Washing Filters for Re-use

10mM Tris-HCl pH8.0 was boiled and poured into a tray containing the filters. This was left at room temperature for thirty minutes. The filters were removed, wrapped in clingfilm and autoradiographed to check that they were clean before re-use.

4.4.5 Storage of filters

Filters were stored at room temperature between sheets of tissue paper.

4.5 DNA Probes

The steps involved in transforming the plasmid DNA into bacteria, growing the bacteria to prepare batches of plasmid and isolating the probe insert from the plasmid are described below in addition to the radioactive labelling of the probe and preannealing.

4.5.1 Transformation of Escherichia coli by Plasmid DNA

Probes were usually received in DNA form. These were introduced into E.coli cells using the calcium chloride method (Maniatis et al, 1982). The first step in the procedure was the preparation of competent cells.

a) Preparation of competent cells

25mls of L broth (appendix 2) was inoculated with one colony of E.coli strain HB101 and incubated at 37°C overnight. 1ml of the overnight culture was added to 100mls of L broth and incubated at 37°C, shaking vigorously, for approximately three hours until the optical density at 550nm was 0.5. The culture was then centrifuged at 1800g for 10 minutes at 4°C and the supernatant discarded. The cell pellet was resuspended in 12.5 mls of ice-cold 50mM CaCl₂, 10mM Tris-

HCl pH8, placed in an ice bath for 30 minutes and then recentrifuged at 1800g for 10 minutes at 4°C. The supernatant was discarded and the cells resuspended in 2 mls of ice-cold 50mM CaCl₂, 10mM Tris-HCl pH8. The cells were dispensed in 0.2ml aliquots into prechilled eppendorfs and stored at 4°C for 12-24 hours to increase their transformation efficiency. The competent cells were then ready for use or storage at -70°C.

b) Transformation

40ng of plasmid DNA in 80µl TE were added to one aliquot of competent cells, mixed and kept on ice for thirty minutes. The eppendorf was then transferred to a water bath at 42°C for two minutes. One ml of L broth was added to the eppendorf and the tube incubated at 37°C for one hour without shaking. 10, 100 and 500µl aliquots of the mixture were spread onto LB agar plates containing the appropriate antibiotic (appendix 2). In addition a control plate was made using untransformed competent cells on an LB agar plate containing the same antibiotic. When the liquid had been absorbed the plates were inverted and incubated at 37°C overnight.

Colonies picked from these plates were grown overnight in LB medium containing the appropriate antibiotic. 0.85 mls of the overnight culture was transferred to a sterile vial containing 0.15 mls of sterile glycerol. After thorough mixing the glycerol stock was stored at -70°C.

4.5.2 Preparation of Plasmid DNA

One colony of transformed cells or a loop from a glycerol stock was inoculated into 10 mls of LB containing the appropriate antibiotic and incubated overnight at 37°C. An aliquot of the overnight culture was added to 250 mls of LB containing the appropriate antibiotic and grown, shaking, at 37°C until the OD₆₀₀ was between 0.4 and 0.8. Chloramphenicol was then added to a final concentration of 150µg/ml and the culture incubated overnight at 37°C with vigorous shaking. The addition of chloramphenicol stops the bacteria dividing but does not stop replication of plasmid DNA therefore the ratio of plasmid DNA to bacterial DNA increases during this time.

The bacterial cells were then harvested by centrifuging the culture at 7000g for 7 minutes at 4°C and discarding the supernatant. The cell pellet was resuspended in 10 mls of solution I (25mM Tris-HCl pH8, 10mM EDTA, 50mM glucose and 5mg/ml fresh lysozyme) and left at room temperature for 10 minutes. 20 mls of freshly made 0.2M NaOH, 1% SDS were then added and the mixture placed on ice for 10 minutes. 10 mls of ice-cold 5M potassium acetate pH4.8 was added and the solution mixed thoroughly and left on ice for thirty minutes. The mixture was centrifuged at 9000g for 10 minutes and then poured through muslin into a measuring cylinder. Cell debris and bacterial DNA were retained

on the muslin. The measured volume was transferred into a clean container and 0.6 volumes of isopropanol added. After 5 minutes at room temperature this was centrifuged at 9000g for 10 minutes at room temperature. The supernatant was discarded and the container left upside down for five minutes to drain. When the pellet was dry it was resuspended in 2.5 mls TE and transferred to a clean universal container. EDTA was added to give a final concentration of 10-20mM and the solution neutralised using 1M Tris base. The volume was made up to 5 mls with TE containing ethidium bromide to give a final concentration of 1mg/ml and then 5.425g CsCl added. The aggregates of ethidium bromide and protein were removed by centrifugation at 20,500g for 10 minutes. The supernatant was put into a Beckman polyallomer tube which was topped up with liquid paraffin. The tubes were balanced to 0.05g and sealed. They were centrifuged for 17 hours at 200,000g at 15°C.

The top of the tube was punctured with a 21-gauge needle and the ethidium stained band of plasmid DNA removed using a needle and syringe. If two bands were seen only the lower band containing the closed circular plasmid DNA was taken. The contents of the syringe were put into a bijou with an equal volume of NaCl saturated isopropanol. These were mixed and then allowed to separate into aqueous and non-aqueous phases. The isopropanol (upper) layer, containing extracted ethidium bromide, was discarded and the process repeated until the aqueous layer was completely clear. The aqueous layer was then transferred to a siliconised corex tube. Two volumes of sterile water and 7.5 times volumes of cold ethanol were added and the tube left at -20°C overnight. The DNA was then precipitated by centrifuging at 14,000g for 10 minutes. The DNA pellet was washed with 70% ethanol to remove salt and then dried in a vacuum dessicator. The DNA was dissolved in 250µl of TE and an aliquot taken for measuring concentration.

3µg of plasmid DNA was digested with endonucleases to cut out the insert. This was then electrophoresed on a 0.8% agarose gel alongside BstEII and HindIII digested lambda to check the size of vector and insert.

4.5.3 Cutting out Inserts

Method 1

The plasmid was digested with the appropriate endonuclease and the insert separated from the vector by electrophoresis through an agarose gel. DEAE cellulose (Whatman DE81) was cut into small pieces the width of the tracks run on the agarose gel and soaked for several hours in 2.5M NaCl. The pieces were then washed several times in distilled water. Prior to use they were soaked for five minutes in electrophoresis running buffer containing 1µg/ml ethidium bromide. The

gel was visualised on an ultraviolet transilluminator and slits were made immediately above and below the insert bands. Pieces of the prepared cellulose paper were inserted into the slits using millipore forceps. The gel was then placed back in the electrophoresis tank and electrophoresis continued for a further thirty minutes. The cellulose strip positioned above the insert band prevents the vector from migrating onto the lower strip along with the insert. The gel was placed back on the transilluminator to check that all of the insert had run onto the paper strips which were then removed. They were placed in a petri dish and viewed on the transilluminator to separate the parts containing DNA from those not containing DNA. The paper was rinsed in distilled water, squeezed dry and placed in 100µl NET (1.5M NaCl, 20mM Tris-HCl pH7.5, 1mM EDTA) in an eppendorf. The tube was incubated for two hours at 37°C. Whilst the sample was incubating a pin hole made in the top and bottom of a 400µl eppendorf and a glass wool plug made in the bottom of it. This small eppendorf was then placed inside a 1.5ml eppendorf from which the top had been removed. The paper and the solution were transferred to the small eppendorf which was then centrifuged for thirty seconds. 2.5 volumes of absolute ethanol was added to the eluate to precipitate the DNA. The sample was placed at 4°C for thirty minutes then centrifuged for thirty minutes. The supernatant was discarded and the pellet rinsed once with 70% ethanol before resuspending it in 25µl TE.

A series of aliquots of known concentration ranging from 10 µg/ml to 100 µg/ml were made using commercial lambda. A piece of parafilm was placed on the ultraviolet transilluminator and 1µl of each of these controls added to 9µl of 2µg/ml ethidium bromide solution. 1µl of the insert was also added to 9µl of the ethidium bromide solution. A photograph was taken and the concentration of the insert compared with the control range. The insert was then diluted to 10µg/ml for use in oligolabelling.

Method 2

The plasmid was digested with endonuclease and the insert separated from vector by electrophoresis through an agarose gel. The insert was then purified from the agarose using a commercial kit "Geneclean" (BIO 10linc.). The band containing the insert was visualised on an ultraviolet transilluminator and excised. The agarose was placed in an eppendorf and broken into pieces using the tip of a pipette. Two to three volumes of saturated sodium iodide solution was added and the eppendorf was incubated at 50°C for 5 minutes or until the agarose was completely dissolved. The "glassmilk" silica matrix was vortexed well before adding 5µl to the eppendorf. The eppendorf was placed on ice for five minutes and then centrifuged for 5 seconds and the supernatant discarded. The pellet was washed three times with "NEW" solution; the pellet was resuspended in 500µl

of ice-cold "NEW" solution, spun for 5 seconds and the supernatant discarded each time. After ensuring that all of the supernatant had been removed the pellet was resuspended in an equal volume of TE and incubated at 50°C for three minutes to elute the DNA from the silica matrix. The tube was centrifuged for thirty seconds and then the supernatant removed and diluted further in TE. After estimating the concentration as described in method 1 it was adjusted to 10ng/μl.

4.5.4 Oligolabelling probes

Labelling solution was prepared by mixing 25μl DTM (25mM dATP, 25mM dGTP, 25mM dTTP, 250mM Tris pH8, 25mM MgCl₂), 7μl OL (1mM EDTA, 1mM Tris pH7.5 containing 90 optical density units/ ml of oligodeoxyribonucleotides) and 25μl 1M Hepes buffer pH6.6.

60ng of probe DNA was sealed in a capillary and denatured by placing in boiling water for two minutes and then cooling to 0°C in an ice bath. The denatured probe was added to an eppendorf tube containing 11.4μl labelling solution, 1μl bovine serum albumin (10mg/ml), 5μl ³²PdCTP (50-100μCi) and 2.5 units DNA polymerase I in a total reaction volume of 25 μl. The reaction was left at room temperature for at least two hours.

The oligolabelled probe was separated from unincorporated DNA on a small Sephadex G-50 column made in a glass pipette. The disintegrations per minute were counted in a Bioscan QC.2000 counter.

4.5.5 Preannealing

Two of the probes, HPRT and p212 (DXS178), were preannealed with sonicated human DNA because they cross hybridised with human repetitive DNA sequences. The labelled probe was placed in an eppendorf in a final concentration of 2.5mg/ml sonicated human DNA and 5 X SSC. The mixture was boiled for 10 minutes to denature the DNA and then incubated at 65°C for thirty minutes to allow the repetitive sequences to anneal. The preannealed probe mixture was then added to hybridisation solution and used to hybridise filters as described above.

The protocol given by Vogelstein with his probe PGK included a preannealing step using sonicated salmon sperm DNA to reduce the background on the autoradiographs. The labelled probe was placed in an eppendorf in a final concentration of 1mg/ml sonicated salmon sperm and 5 X SSC. The mixture was boiled for 10 minutes to denature the DNA and the incubated at 65°C for thirty minutes prior to use.

4.6 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis requires high molecular weight DNA. DNA prepared by normal methods undergoes shearing during the pipetting procedures and therefore is not satisfactory for this technique. DNA for pulsed field gel electrophoresis is prepared in agarose blocks as described below. In addition when handling the agarose blocks or any of the solutions contamination by nucleases or physical damage will result in smaller DNA. All solutions used were sterile. Blocks were manipulated using glass pipettes which were sterilised by holding in a flame which also allowed the end to be sealed and a hook to be formed. When blocks had to be moved they were usually cooled to 4°C first as this made them more rigid and less likely to be broken.

4.6.1 Preparation of DNA from Lymphocytes

High molecular weight DNA was prepared in agarose blocks from fresh lymphocytes and EBV transformed cells. Lymphocytes were prepared from blood samples taken into preservative free heparin by separation on a Ficoll-Hypaque gradient as described in section 4.7.1. EBV transformed cells were taken during active cell division i.e. 24-48 hours after splitting as overconfluent cells contain more mitochondrial DNA. They were also separated on a Ficoll-Hypaque gradient to remove any dead cells and debris and so reduce the amount of degraded DNA in the final sample.

Cells were suspended in sterile PBS at 14.3×10^6 per ml so that when the blocks were formed there were 0.5×10^6 diploid cells per block. As each haploid cell contains 6 pg DNA this is equivalent to 3µg per block. The same amount of DNA was used per block throughout all the experiments as it has been reported that migration is faster with a lower concentration of DNA and that excessively overloaded lanes hardly focus at all.

The blocks were made in a perspex block formers (LKB). The base of these were sealed with tape and they were precooled on ice. The cell suspensions were mixed with an equal volume of 1% ultra-pure low melting point agarose in PBS which had been melted and then kept at 42°C. 70µl aliquots of the mixture were immediately dispensed into the wells of the block former. Precooling the block former helped the agarose to set quickly before the cells settled so that the DNA was evenly distributed throughout the block. The block formers were then left at 4°C for 45 minutes. At the end of this time the tape was removed from the bottom of the block former and the blocks gently tapped out onto clean clingfilm and transferred to falcon tubes for protein digestion.

Ten agarose blocks were added to 2.5 mls of 0.5M EDTA pH8, 1% SDS and 2mg/ml proteinase K in each Falcon tube. This was incubated

for 48 hours at 50°C, inverting the tube occasionally. The blocks were then washed to remove the protein digestion buffer by adding TE buffer to the top of the Falcon tube allowing the blocks to sink to the bottom and decanting the TE. This was repeated three times and the tube then filled with TE plus 0.04 mg/ml PMSF (phenylmethylsulphonylfluoride, freshly prepared in anhydrous isopropanol) and incubated for 30 minutes at 55°C. The fluid was decanted and this last step repeated. The blocks were then ready for use or storage in 0.5M EDTA pH8 at 4°C. Blocks stored in this way were washed twice for thirty minutes in TE to remove the EDTA prior to digestion.

4.6.2 Restriction Enzyme Digestion

Restriction enzyme digests were carried out in 1.5 ml eppendorf tubes. All the solutions including the enzyme were placed in the tube and mixed by vortexing before the addition of the block. 20 units of enzyme were used for digestion of each block following the buffer and temperature conditions recommended by manufacturer but adding 100µg of bovine serum albumin. The final volume including the 75µl agarose block containing the DNA was 200µl. Sfi partial digests were generated by stopping digestion after fifteen, thirty or fortyfive minutes by addition of 10µl of 0.5M EDTA and placing the eppendorf on ice. NotI digests were done overnight. To generate double digests digestion was carried out with one enzyme first, the agarose block was then tipped into a plastic weighboat and washed with sterile water and then added to an eppendorf containing the second buffer and enzyme. With the exception of deliberate partial digests and NotI digests the blocks were digested for 4 hours in each enzyme.

4.6.3 Preparation of the Gel and Electrophoresis

LKB pulsed field gel electrophoresis apparatus was used with the clamped homogeneous electric field electrode array. Two litres of $\frac{1}{2}$ x electrophoresis buffer was put into the electrophoresis tank and precooled for 2 hours before starting electrophoresis, with the cooling system set to 8°C. The concentration of the electrophoresis buffer was reduced because using standard electrophoresis buffer the current rose during the run and invariably became a limiting factor resulting in a drop in voltage. Using 20mM Tris, 10mM sodium acetate, 1mM EDTA the current rose only a small amount during the run, never becoming the limiting factor and so conditions could be reliably reproduced.

A 1% agarose gel in $\frac{1}{2}$ x electrophoresis buffer was poured in the LKB mould and precooled to 4°C. The eppendorfs containing the digested DNA blocks were placed on ice for thirty minutes. The blocks were then tipped out into clean weigh boats. The blocks were manipulated into

the wells of the gel using glass pipettes as described above. *Saccharomyces cerevisiae* markers, made by M O'Reilly, were put into the wells at each end of the DNA tracks and, depending on the number of lanes run, a marker track was also run in the middle of the gel. The loaded gel was then submerged in the electrophoresis buffer in the tank. The switch times and length of run were adjusted to give maximal separation in different size ranges.

4.6.4 Staining the Gel

The gel was stained with 20µg/ml ethidium bromide in running buffer for twenty minutes and then destained in running buffer for at least one hour with one change of buffer. The gel was placed next to the ruler on the ultraviolet transilluminator and photographed.

4.6.5 Transfer of the DNA

Large DNA fragments will not transfer and therefore the very large fragments of DNA in the pulsed field gel had to be nicked before transfer. This was done by placing the gel in 240mM HCl for twenty minutes (acid depurination). The gel was then rinsed in denaturing solution and then denatured and neutralised using the standard method (section 4.4.1). The blot was set up as shown in figure 4.1 but transfer continued for 36 hours changing the towels several times during the process. The filter was then marked and baked for two hours at 80°C.

4.6.6 Prehybridisation and Hybridisation of Filters

Initially the hybridisation solution and conditions described in section 4.4.2 were used. However, it was found that the addition of dextran sulphate at a final concentration of 10% in the hybridisation solution improved the band intensity of the autoradiographs.

4.6.7 Washing Filters

The filters were washed using the protocol described in section 4.4.3. After use filters were stripped as described in section 4.4.4 and stored at room temperature between sheets of tissue paper until re-probing.

4.7 Preparation of Cells

4.7.1 Purification of Mononuclear Cells

Heparinised venous blood was diluted with an equal volume of RPMI 1640 supplemented with 25mM Hepes. This was layered onto Ficoll-Hypaque (specific gravity 1.077) in a 2:1 ratio. The sample was centrifuged at 1250g for 20 minutes at room temperature with the centrifuge break off. Mononuclear cells were collected from the interface and made up to an equal volume with RPMI 1640 then centrifuged at 200g for 15 minutes at room temperature. The pellet was gently resuspended and washed twice in RPMI 1640, 25 mM Hepes and 5% fetal calf serum. Cell viability was assessed by trypan blue exclusion.

4.7.2 Purification of T Lymphocytes

Peripheral blood mononuclear cells were resuspended at $5 \times 10^6/\text{ml}$ and 0.5mls of 10% S-2-aminoethylisothiuronium bromide hydrobromide treated sheep red cells (Kaplan and Clark, 1974) were added to each 2 mls of mononuclear cells and the fetal calf serum concentration increased from 5% to 15% to prevent the rosettes clumping. The tube was centrifuged at 200g for 15 minutes with the brake off and then placed on ice for one hour. The rosettes were then resuspended by gentle rotation of the tube and the cell suspension layered onto Percoll (specific gravity 1.080) in a 2:1 ratio. The sample was centrifuged at 1250g for twenty minutes at room temperature with the brake off and then the interface containing the non-rosette forming cells, the E⁻ fraction, was removed. The remaining Percoll layer was aspirated, taking care not to disrupt the red cell pellet, and discarded. The pellet was resuspended in 5mls of Gey's solution for one minute (appendix 2) and immediately after red cell lysis diluted with wash medium. The E⁻ cells were resuspended in wash medium and both the E⁺ and E⁻ cells were washed twice. Cell purity was assessed by monoclonal antibody staining and viability by trypan blue exclusion.

4.7.3 Purification of Granulocytes

Granulocytes were purified from the red cell pellet remaining after Ficoll Hypaque separation for the purification of mononuclear cells. An equal volume of RPMI 1640 was added to the pellet followed by an equal volume of Dextran sulphate. These were mixed by inversion and left at room temperature for 40 minutes to allow red cell sedimentation. The upper phase containing the granulocytes was then aspirated, and washed three times.

4.7.4 Counting Cells

An aliquot from the cell suspension was mixed with an equal volume of trypan blue. The number of viable cells was counted by trypan blue exclusion on a haemocytometer.

4.7.5 Monoclonal Antibody Staining

The purity of the E positive cells, E negative cells and granulocytes was assessed using the monoclonal antibodies B1(CD20), UCHM1(CD14), T3(CD3), HNK1 and TG1(CD15). This was carried out by staff in the clinical immunology laboratory.

4.7.6. Establishing Epstein-Barr Virus Transformed B Cell Lines

5-20 X 10⁶ mononuclear cells or E⁻ cells were incubated with 1ml of supernatant from the EBV secreting B958 marmoset line for 1 hour at 37°C for 1 hour. The cells were then washed twice resuspended at 10⁶ cells/ml in culture medium (appendix 2) and 1µg/ml cyclosporin A and dispensed into the wells of a 24-well Costar plate. The outer wells of the plate were filled with sterile water to prevent evaporation of the culture medium. The plates were incubated at 37°C in 5%CO₂ in air. The cell lines were expanded into culture flasks when established.

4.8 Data Analysis

Data was prepared for analysis using LINKSYS (Attwood and Bryant, 1988). Two point linkage was carried out using LIPED (Ott, 1974). Multipoint linkage analysis was carried out using the LINKMAP section of LINKAGE version 4.7 (Lathrop et al, 1984).

The output files from LINKMAP analysis give information about points on the map in the form of $-2 \ln$ likelihoods. To generate location scores from these the $-2 \ln$ likelihood at 0.5 recombination from the markers at the left and right of the fixed map is subtracted from the $-2 \ln$ likelihood at the point of interest. The figure is divided by 2 to give the \ln likelihood. In order to convert the resulting number to a \log_{10} location score it is divided by 2.303.

CHAPTER 5. Mapping Results

5.1 Ordering Probes on the Long Arm of the X Chromosome

5.1.1 Families

Recombinations were noted on proximal Xq in four families. These four families were used as a reference panel for ordering probes between Xp11 and Xq22. Family MAN had been collected for linkage studies to localise Bruton's agammaglobulinaemia and was included in this study after a recombination was observed between pDP34 and the disease locus in one of three affected sons. Family BER had been collected for linkage studies to localise severe combined immunodeficiency and was included in this study after observing a crossover between the disease locus and 19.2. Family LIT was referred for the prenatal diagnosis of X-linked ectodermal dysplasia and included in this study after a recombination was observed between the disease locus and the most closely linked probe at the time of referral, pDP34 (MacDermot et al, 1986). Family HIS was also referred for genetic counselling. The affected males had choroideremia, mental retardation and additional neurological findings. They were included in this study after a recombination was observed between the disease locus and pDP34 which is closely linked to choroideremia.

5.1.2 Probes

These families were investigated using a panel of 14 DNA markers detecting restriction fragment length polymorphisms between Xp11 and Xq22 (Table 5.1).

Locus	Probe Name	Regional Assignment
DXS146	pTAK8	p11-q12
DXS1	p8	q11-q13
DXS159	cpX289	p11.1-q21.3
PGK1	pSPT/PGK	q13
DXS106	cpX203	p11-q21.3
DXS72	pX65H7	q13-q22
DXYS1	pDP34	q13-q21.1
DXS3	p19.2	q21.3-q22
DXS94	pXG12	q11-q13
DXS178	p212	q11-qter
DXS17	S21/S9	q21.3-q22
DXS87	pA13.R1	q21-q24
DXS88	G3-1	q11-q22
DXS255	M27 β	p11.3-centromere

Table 5.1 Regional assignments of the probes based on information available at the start of the study (Eighth Human Gene Mapping Meeting). M27 β (DXS255) was described after this Meeting (Fraser et al, 1987). Details of the polymorphisms are given in Appendix 1.

5.1.3 Analysis of Recombinant Chromosomes

Cosegregation of alleles was used to place probes above or below the site of the crossover. There were at least 11 crossovers observed on 10 recombinant chromosomes. No linkage analysis was carried out because the sample was selected for recombinations and therefore biased.

The probes p8, pTAK8, pXG12 and the PGK1 locus were mapped proximal to the XY homologous region, pDP34 within it and 19.2, S21, pA13.R1 distal to it at the Eighth Human Gene Mapping Meeting (Human Gene Mapping 8, 1985). The localisation of pXG12 was revised to Xq21.3-q22, distal to the region of homology, prior to this study (Malcolm et al, 1987). cpX289, cpX203, pX65H7, pG3-1 and p212 were not localised proximal or distal to the region of XY homology at the Eighth Human Gene Mapping Meeting. Table 5.2 is a summary of the results from the 10 chromosomes where recombinations were observed within haplotypes. It shows the number of recombinations and the number of informative meioses for cpX289, cpX203, pX65H7, pG3-1, p212 and pXG12 against probes on proximal Xp, and probes on the long arm proximal to, within and distal to the XY homologous region. M27 β was used as the short arm probe. Five of the 10 recombinant chromosomes gave information for p8 and six for PGK. As there were no recombinations between PGK and p8 they are considered as a haplotype for the region proximal to the homologous region. pDP34 was chosen as the probe within the region of homology. 19.2 was chosen as the probe distal to the region of XY homology.

	M27 β	PGK and p8	pDP34	19.2
cpX289	0/7	0/6	3/9	5/7
pX65H7	0/5	0/6	2/7	4/5
cpX203	0/4	1/4	3/5	5/6
p212	2/2	2/2	1/2	1/2
G3-1	1/2	-	0/2	1/2
pXG12	1/1	1/2	1/2	0/1

Table 5.2 Recombinations observed between unassigned probes and a probe on the short arm of the X chromosome, and probes on the long arm localised proximal, within or distal to the XY homologous region. The number of informative meioses is given as the denominator.

The probes are plotted as haplotypes (figure 5.1) in the order giving the least number of recombinations per chromosome. The probes are placed proximal or distal to the region of XY homology based on the HGM8 assignments for pTAK8, p8, PGK1, 19.2, S21, and pA13.R1 and using the results from table 5 for cpX289, cpX203, pX65H7, p212, pG3-1 and pXG12.

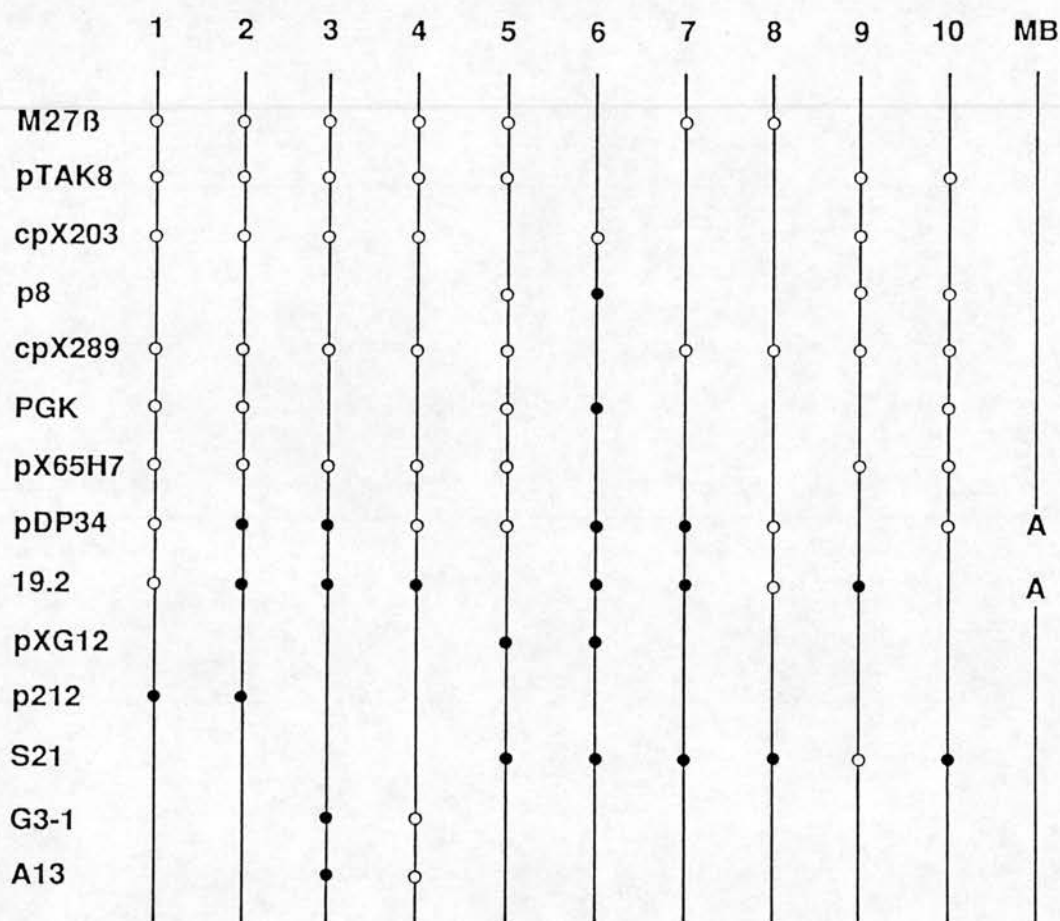


Figure 5.1 Diagrammatic representation of the recombinant chromosomes. The probes are shown on the left hand side. Informative loci are represented by circles. Open circles correspond to loci derived from one maternal X chromosome and closed circles correspond to the other maternal X chromosome. Chromosome MB shows the deletion of pDP34 and 19.2 in patient MB who has choroideremia. A indicates absence of probe hybridisation to DNA from patient MB.

In these four families no recombinations were observed between M27 β , pTAK8, p8, cpX289, pSPT/PGK or pX65H7. There is one recombination between cpX203 and pSPT/PGK and p8 consistent with cpX203 being proximal to p8 (recombinant chromosome 6). The meiosis leading to this recombinant chromosome was not informative for cpX289, pX65H7, pTAK8 or M27 β .

The recombination on recombinant chromosome 1 (figure 5.1) places p212 distal to 19.2. There is no information however about the order of p212 relative to pXG12 or S21. Neither is there any information about the order of pXG12 relative to 19.2 or S21.

The two recombinations between 19.2 and S21 do not resolve the order of the two probes. One of the recombinant chromosomes (recombinant chromosome 8 in figure 5.1) is also informative for pDP34 which segregates with 19.2 but not S21 suggesting that S21 is distal to 19.2. The second chromosome with a recombination between 19.2 and S21 is not informative for pDP34 (recombinant chromosome 9 in figure 5.1). With either order there would be two crossovers on one of the recombinant chromosomes.

Two meioses gave information about the position of G3-1, recombinant chromosomes 3 and 4 in figure 5.1. Neither of these meioses were informative for pXG12, S21 or p212. On recombinant chromosome 3 the crossover occurs between pX65H7 and the XY homologous region. G3-1 segregates with the XY homologous region and 19.2 on this chromosome and recombines with pX65H7 and more proximal probes. On recombinant chromosome 4 there is a crossover between 19.2 and G3-1 with G3-1 segregating with all the informative probes in and proximal to the XY homologous region. This data places G3-1 between pX65H7 and 19.2.

5.1.4 Deletion Mapping

Family MB was referred for prenatal diagnosis. The affected male had choroideremia and mental retardation. His mother and sister had been seen by an ophthalmologist and found to have the clinical features of carriers of choroideremia. On investigation the X chromosome allele of pDP34 was deleted in the affected male. Both a Y specific and an X chromosome specific pDP34 allele was present in the fetus. When examined at eighteen months of age the child was developing normally and had no abnormalities on ophthalmoscopy.

The affected male was investigated using the panel of X linked probes. He was deleted for pDP34 and 19.2 but not for any of the other probes. (Figure 5.1)

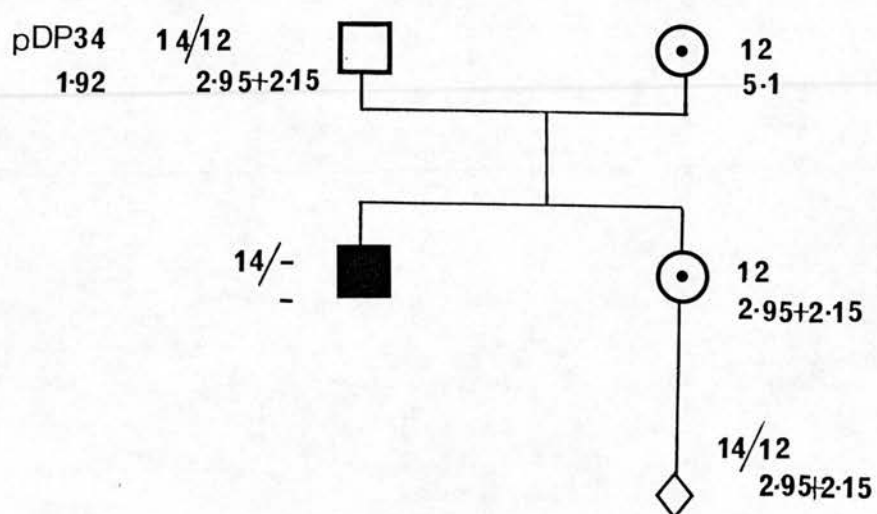


Figure 5.2 Pedigree MB showing the pDP34 and 19.2 alleles. I₂ and II₂ are hemizygous for both probes.

5.1.5 pG3-1 and pA13.R1

These probes were isolated from a λ gt wes 19/20 flow sorted library. pG3-1 is a 1.0kb EcoRI/BamHI fragment cloned into pBR328 and pA13.R1 is a 1.6kb EcoRI fragment cloned into pBR328. In the original description they were given different physical localisations and different allele frequencies (Upadhyaya et al, 1986). It was noted when rehybridising a nylon filter that the upper allele detected by the probes was in the same position at 4.7kb. The lower allele was 3.5kb for pG3-1 and 1.1kb for pA13.R1. The sum of the two lower alleles is 4.6kb, approximately the size of the upper allele for both probes. pA13.R1 detects a constant band at 5.4kb in addition to the polymorphic bands. When these probes were hybridised to a filter with BamHI digested DNA both hybridised to the same band. In addition complete disequilibrium was noted between the alleles. These two probes therefore detect the same BglII polymorphism (figure 5.3).

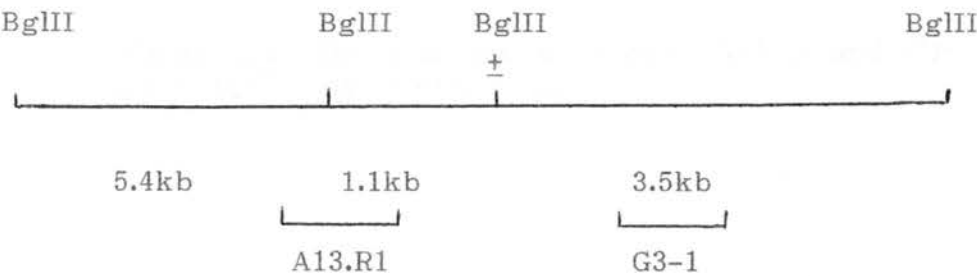


Figure 5.3 The Position of Probes A13.R1 and G3-1 Relative to the Same BglII Polymorphic Site.

5.1.6 Pulsed Field Gel Electrophoresis Results

The pXG12, p212 and S21 bands generated by hybridising filters with DNA digested with Sfi, NotI, BssHII and Sfi/NotI, Sfi/BssHII double digests are listed in table 5.4. A photograph of one of the gels used in obtaining this information and the corresponding autoradiographs are shown in figure 5.4.

	Sfi	Sfi/NotI	NotI	Sfi/BssHII	BssHII
pXG12	295 620 650 710	295	LM	210 295	425 LM
p212	180 400	180 LM	LM	180	505 LM
S21	170 230 295 390 465 LM	170 230 295	LM	170 230 295 390	LM

Table 5.4 Band sizes in kilobases detected by the probes pXG12, p212 and S21. LM indicates hybridisation to the region of limiting mobility.

The band sizes recorded for the Sfi partial digests were generated by rehybridisation of filters with the three probes. Differences between the results are, therefore, not due to different degrees of Sfi digestion or differences in calibration of filters. The bands detected by the probes do not coincide at the top of the ladders. These probes have not been physically linked using Sfi partial digests.

All three probes hybridised to the limiting mobility region in the NotI tracks. The probes also hybridised to the limiting mobility in the BssHII tracks but, in addition, a 425kb band was detected by pXG12 and a 505kb band by p212. The bands seen in the Sfi/NotI double digest tracks were Sfi bands, no new bands were generated by these digests. The bands detected by p212 and S21 in the Sfi/BssHII double digests were Sfi bands. A new band of 210kb was detected by pXG12 in the Sfi/BssHII double digest.

Hybridisation of the filter from the gel shown in figure 5.3 with these three probes did not give a clear band in the Sfi/HpaII track. In all three cases a smear was seen of less than 120kb.

The total length of the Sfi bands detected by the probes was 1.575 megabases. Adding the BssHII bands onto the restriction map gives a total length of 1.895 megabases detected without linking any of the three probes.

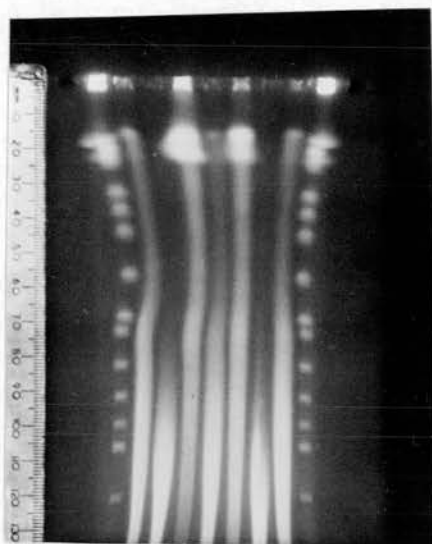


Figure 5.4a Photograph of Ethidium Stained Gel following 39 hour electrophoresis at 170 volts using the CHEF electrode configuration, buffer temperature 12.5°C, switch time 70 seconds. From left to right the tracks are marker, Sfi partial, Sfi/NotI, NotI, Sfi/BssHII, BssHII, Sfi/HpaII, Sfi partial and marker. The *S.cerevaciae* yeast markers were made from strain YP148 by M O'Reilly.

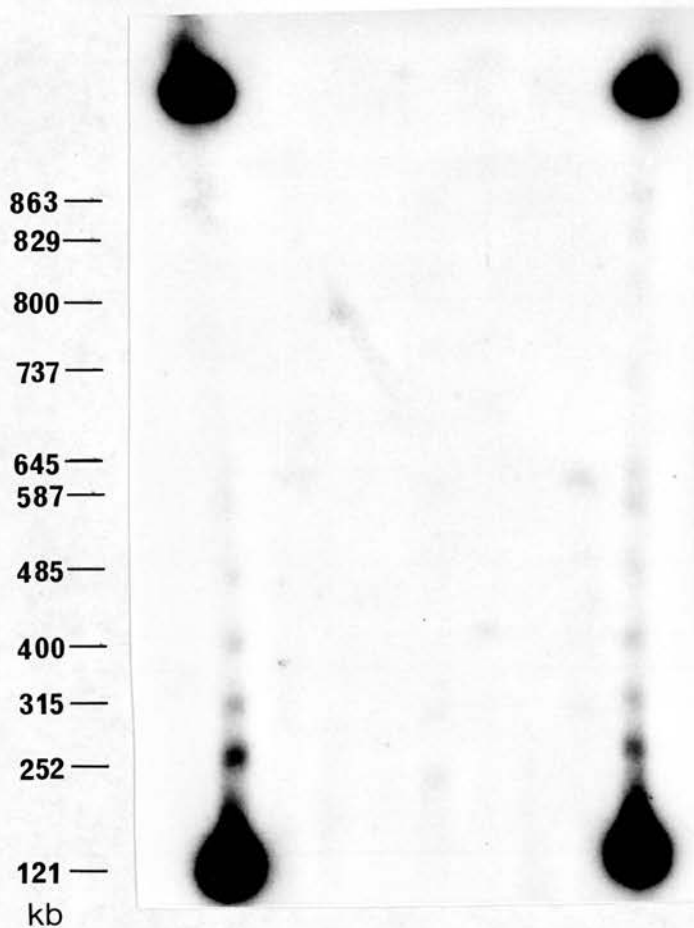


Figure 5.4b Autoradiograph of the filter from the gel in figure 5.4a hybridised with pXG12. The position and size of the yeast bands are marked on the figure. The smallest band using this strain is a fragment of *S.cerevaciae* chromosome 7. The chromosome sizes were calculated by D.Williamson, National Institute for Medical Research, Mill Hill (personal communication)

The 620 kb Sfi band, 425kb BssHII band and the 295kb and 210kb Sfi/BssHII bands can be seen on this autoradiograph.

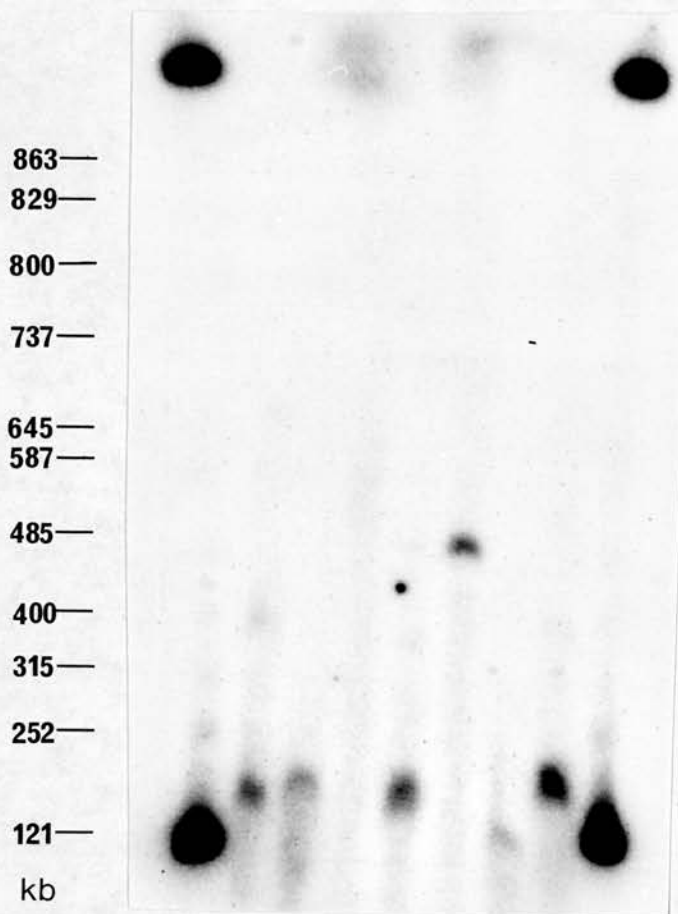


Figure 5.4c Autoradiograph of the filter from the gel shown in figure 5.4a hybridised with p212. The position and size of the yeast bands are marked on the figure. The smallest band using this strain is a fragment of *S.cerevaciae* chromosome 7. The chromosome sizes were calculated by D.Williamson, National Institute for Medical Research, Mill Hill (personal communication)

The 505kb BssHII band and the 400kb and 180kb Sfi bands can be seen on this autoradiograph.

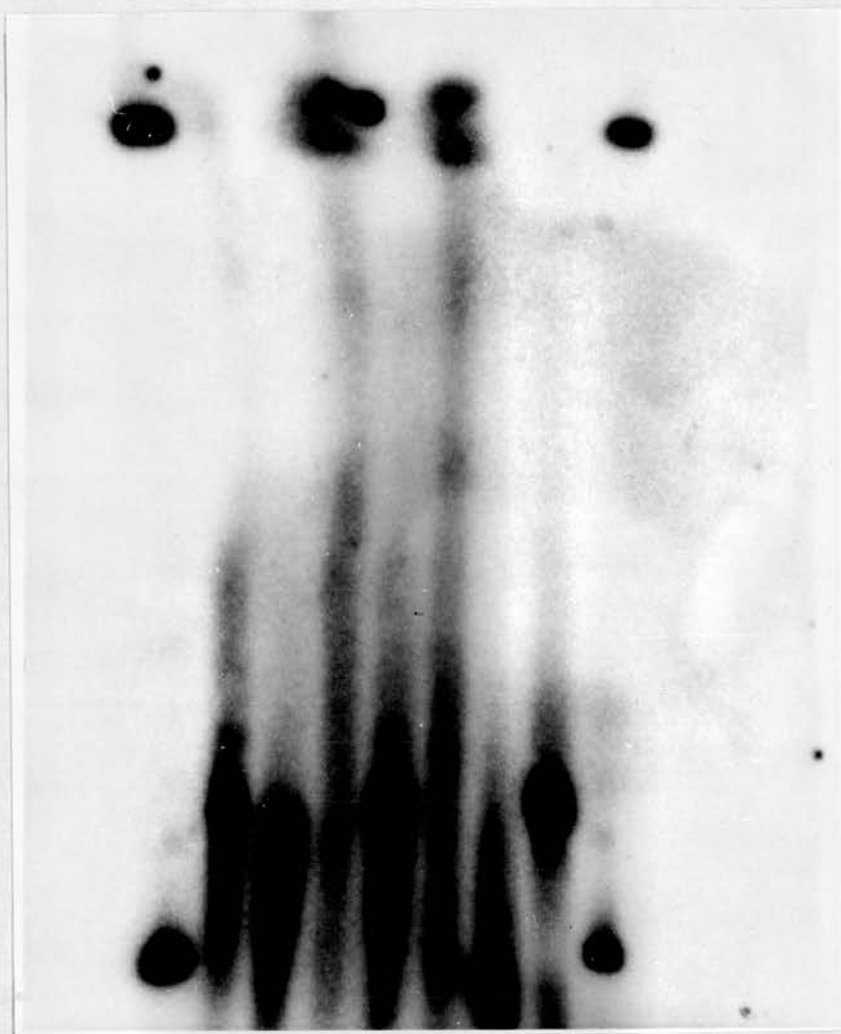


Figure 5.4d Autoradiograph of the filter from the gel shown in figure 5.4a hybridised with S21. The bands on this autoradiograph were not clear enough to give accurate size estimations. The S21 band sizes in Table 5.4 were based on hybridisation of filters from different gels.



5.2 Localisation of X-linked Diseases

The clinical features and investigation of two of the families that had recombinations on the long arm of the X chromosome and were used in the probe ordering study are described in detail below. In these two families, which were clinical referrals, the results of DNA investigation did not fit with the available information about the disease localisation.

5.2.1 Choroideremia

Family HIS was referred for genetic counselling. The three males in generation II had the ophthalmological findings of choroideremia. The notes from the referring hospital state that all three had mental retardation, in addition II₂ had signs of mild spastic cerebral palsy and II₃ had cerebellar ataxia, dysarthria and nystagmus. The diagnosis of choroideremia had been confirmed by ophthalmologists at The Hospitals for Sick Children, Great Ormond Street. Unfortunately the family did not wish the affected males to be re-examined at the time of referral for genetic counselling. The fundi of both of the females in the pedigree had the classical appearance found in carriers of choroideremia. Neither of the women had mental retardation or abnormal neurological findings.

They were investigated using the panel of DNA probes in table 5.1. The mother, I₂, was informative for only five of the fourteen probes - M27 β , cpX289, pDP34, 19.2 and S21. As both mother and daughter were heterozygous for cpX289, pDP34, 19.2 and S21 the haplotype in II₄ was deduced from the paternal haplotype. Paternity was checked using p λ g3 (Jeffreys et al, 1988).

The disease did not segregate with any of these five informative probes in the three affected males. The results were analysed using the LINKMAP section of LINKAGE 4.7. The probability of the disease locus being between the probes cpX289, pDP34 and 19.2 was considered using a distance of 8 cM between cpX289 and pDP34 and 4.4 cM between pDP34 and 19.2 (Arveiler et al, 1987). The results are shown in figure 5.6. The maximum lod score between cpX289 and pDP34 was -1.85 and the maximum lod score between pDP34 and 19.2 was -2.39.

Cytogenetic analysis in the three males failed to reveal a microscopical deletion on the X chromosome. All fourteen probes tested gave a hybridisation signal with DNA from the affected males. Following a report that plbD5 (DXS165) failed to hybridise to DNA from two out of eight males with choroideremia (Cremers et al, 1987) a filter of DNA samples from the three males was sent to H-H Ropers who kindly hybridised it with this probe. This sequence and the probes pJL8 and pJL68, which were generated by reassociation techniques from a patient with a deletion of the X chromosome and choroideremia (Nussbaum et al, 1987), hybridised to the DNA of all three males.

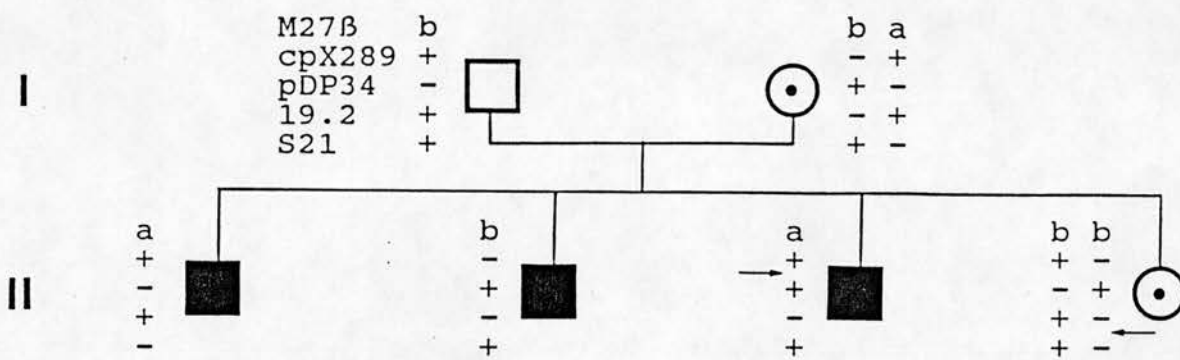


Figure 5.5 Pedigree HIS showing haplotypes for the probes M27 β , cpX289, pDP34, 19.2 and S21. + indicates presence of a polymorphic site and - indicates absence of the polymorphic site. Recombinations are marked by an arrow.

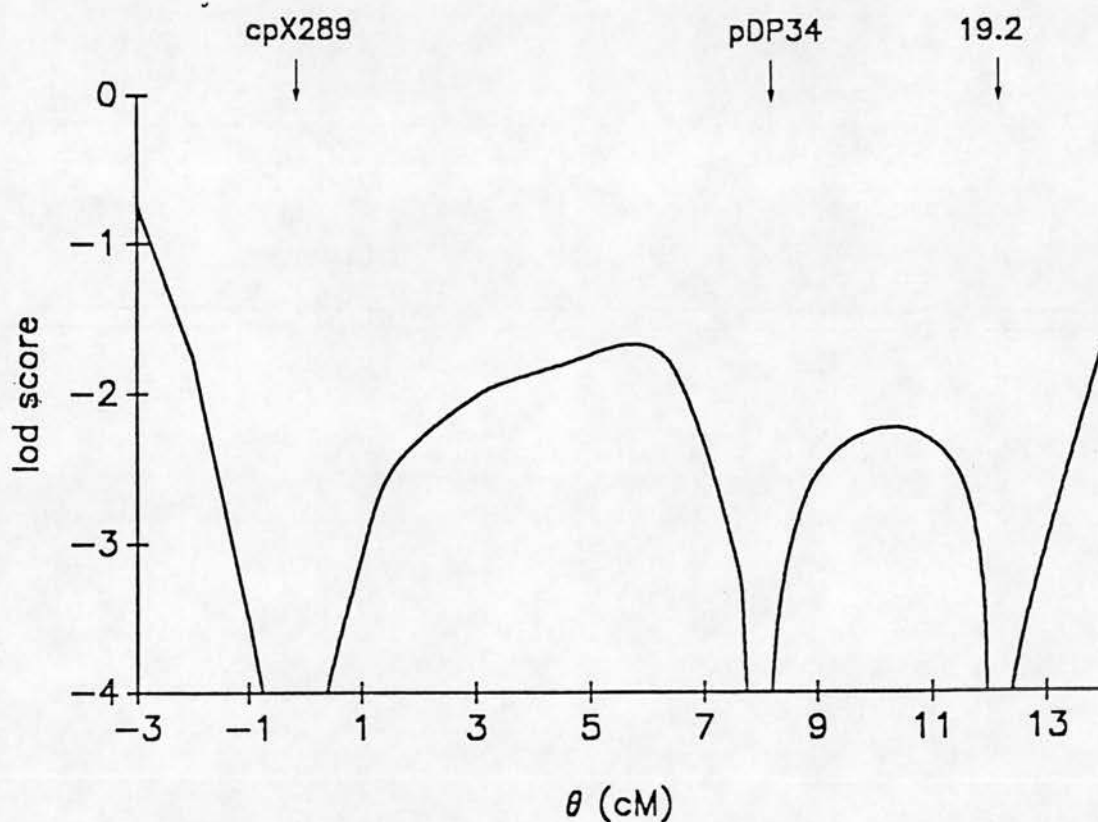


Figure 5.6 Multipoint linkage analysis using LINKMAP to calculate the probability of the disease locus in pedigree HIS lying between cpX289 and 19.2 using a framework of cpX289, pDP34 and 19.2. A recombination distance of 8 cM was used between cpX289 and pDP34, and a recombination distance of 4.4 cM between pDP34 and 19.2.

5.2.2 Hypohydrotic Ectodermal Dysplasia

Family LIT (figure 5.7) was referred for predictive testing for ectodermal dysplasia. The affected male III₁ had few conical shaped teeth and fine sparse hair. He had periorbital pigmentation but his nasal bridge appeared normal. His nipples were absent. The distribution of subcutaneous fat was normal. His parents gave a history of poor sweating and heat intolerance. A whole back starch and iodine sweat test was performed and sweat pores were present over most of his back but absent from the nape of his neck. Fingertip impressions were made in dental impression material. The sweat pore count was normal and the dermatoglyphic pattern was well preserved. In addition to the findings which led to the diagnosis of hypohydrotic ectodermal dysplasia he had reflux nephropathy secondary to ureteric valves and had a unilateral nephrectomy because of this.

His mother lacked both mandibular first premolars and the left lateral incisor in the maxilla. Her right lateral incisor in the maxilla was abnormally pointed and both mandibular incisors were small. She had pale, fine hair and was noted to have an accessory nipple. Both individuals in generation I were reported to be normal but not examined.

Subject II₂ lacked both upper lateral incisors. She had pale, fine hair and eczema. The third sister, II₃, lacked a right upper lateral incisor and her left upper lateral incisor had been extracted because it was small and pointed. Her appearance differed from her two sisters as she had thicker, dark hair. Her sons, dizygotic twins, have no features of the condition.

A whole back sweat test was carried out on all three sisters and was normal. Dental X rays were examined from all four women. There was definite hypodontia in subject II₁ and a suggestion of hypodontia in subjects I₂, II₂ and II₃.

The referral diagnosis of HED was confirmed in the affected boy. Taking two missing or abnormally shaped teeth as being diagnostic of carrier status when there is a family history, all three sisters were diagnosed as carriers. The accessory nipple in subject II₁ was further evidence that she is a gene carrier. Blood samples were taken for DNA extraction and analysis.

The results are shown on the pedigree in figure 5.6. The order of the probes on which the haplotypes are based is (M27 β - pTAK8) - cpX203 - p8 - cpX289 - pSPT/PGK - pX65H7 - pDP34 - 7b - 19.2 - pXG12 - S21 - G3-1. This order is based on studies of somatic cell hybrids, deletions (Arveiler et al, 1987; Mahtani, Willard, 1988) and results in this thesis. Recombinations have occurred within this haplotype in four out of six meioses in females. In the meiosis leading to II₁ a recombination has occurred between pX65H7 and pDP34. In the meiosis leading to II₃ a recombination has occurred between pDP34 and 19.2 and

5.2.3 Severe Combined Immunodeficiency

Five families with XSCID were investigated using the probes cpX203, p8, cpX289, pSPT/PGK, pX65H7 and pDP34. The families and the haplotype results are shown in figure 5.8. The order of the probes in the haplotypes has been established by studies of somatic cell hybrids derived from females with X;autosome translocations (Arveiler et al, 1987), males with X chromosome deletions (Schwartz et al, 1988) and the analysis of recombinant chromosomes presented in this thesis. The haplotype for subject I₂ of pedigree HAL is ambiguous but there are no crossovers between the five informative probes in any of the possible haplotypes.

There were no recombinations between XSCID and p8, cpX289, pSPT/PGK or pX65H7. Calculating two point lod scores using obligate carrier females and their male offspring only, the maximum two point lod scores between XSCID and p8, cpX289, pSPT/PGK, and pX65H7 were 1.7, 2.61, 1.63 and 2.31 respectively, all at $\theta = 0$. Including females whose carrier status had been established by X inactivation patterns the maximum two point lod scores between XSCID and p8, cpX289, pSPT/PGK, and pX65H7 were 1.7, 3.21, 3.01, and 2.91 respectively, all at $\theta = 0$.

A recombination was observed between the disease and cpX203 in subject II₂ of pedigree TEB and between the disease and pDP34 in subject IV₁ of pedigree KHA. In the first the recombination occurred between cpX203 and cpX289, the meiosis was not informative for p8. In the second the recombination occurred between pDP34 and cpX289, the meiosis was not informative for pSPT/PGK and unfortunately no result was obtained for pX65H7 for this individual.

LINKMAP analysis was carried out using the probes cpX203, cpX289, pSPT/PGK and pDP34. Females whose carrier status had been established by X inactivation patterns were included in the analysis. There is no figure for the cpX289-cpX203 distance. The p8-cpX289 distance is 1 cM (Arveiler et al, 1987) and p8 is between cpX203 and cpX289. The cpX289-cpX203 distance used in the analysis was 2 cM. The distance estimated between cpX289 and pDP34 is 8 cM (Arveiler et al, 1987). There is no published estimate of the distance between cpX289 and pSPT/PGK or pSPT/PGK and pDP34. Three analyses were carried out using recombination fractions 0.02 0.01 0.07, then 0.02 0.04 0.04 and finally 0.02 0.07 0.01 so calculating the lod score for varying positions of pSPT/PGK between cpX289 and pDP34. The maximum lod scores using LINKMAP were 5.11 at cpX289 and 5.03 at pSPT/PGK in the first analysis (figure 5.9). The lod score at pSPT/PGK falls to 4.7 in the second analysis and 4 in the third analysis with pSPT/PGK placed 7 cM from cpX289 and only 1 cM from pDP34.

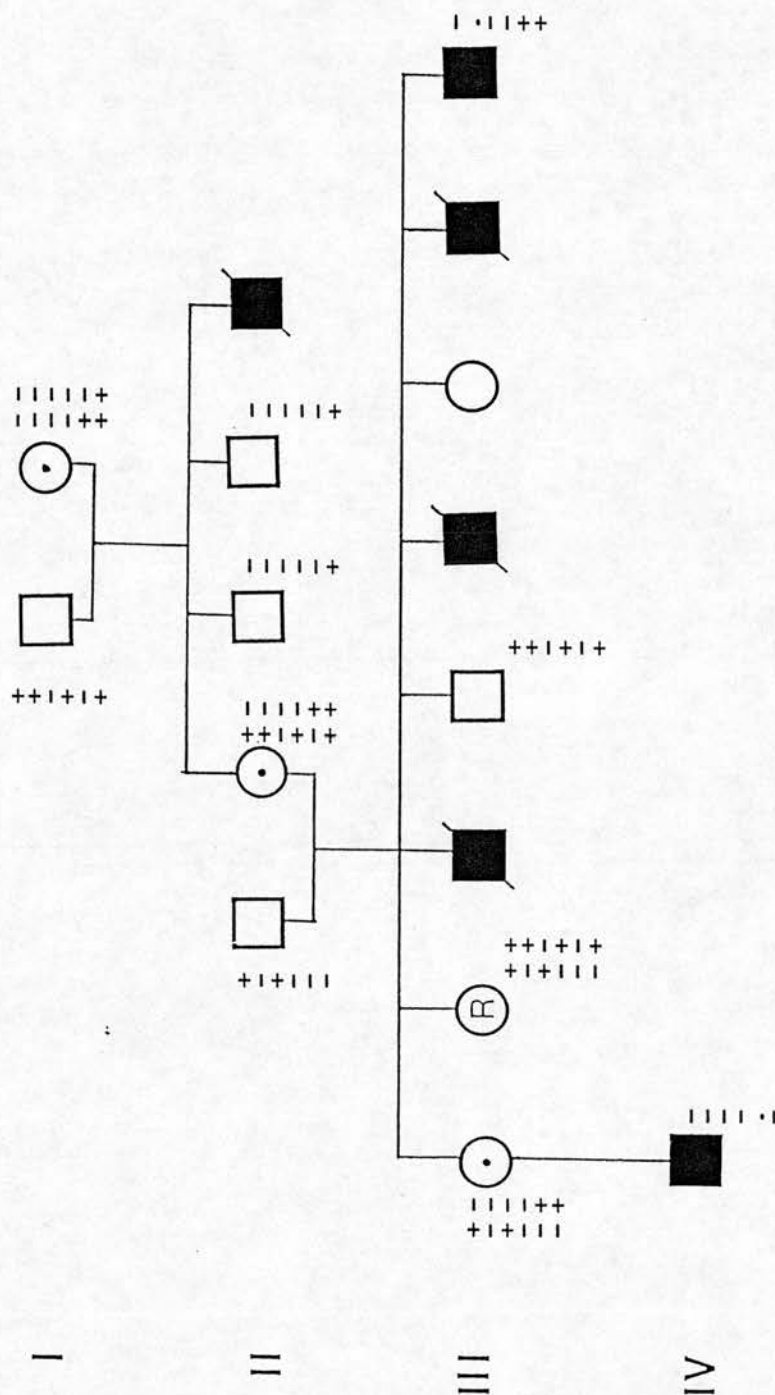


Figure 5.8a Pedigrees with haplotypes of the five families with XSCD. + indicates presence of a polymorphic site and - indicates its absence. The probe order on the pedigrees is cpX203, p8, cpX289, pSPT/PGK, pX65H7, pDP34. Obligate carrier female. (R) female with a non-random pattern of X inactivation. (O) female with a random pattern of X inactivation. (•) female of unknown carrier status, homozygous for the BstXI polymorphism detected by pSPT/PGK. • result not obtained. Recombinations are marked by arrows.

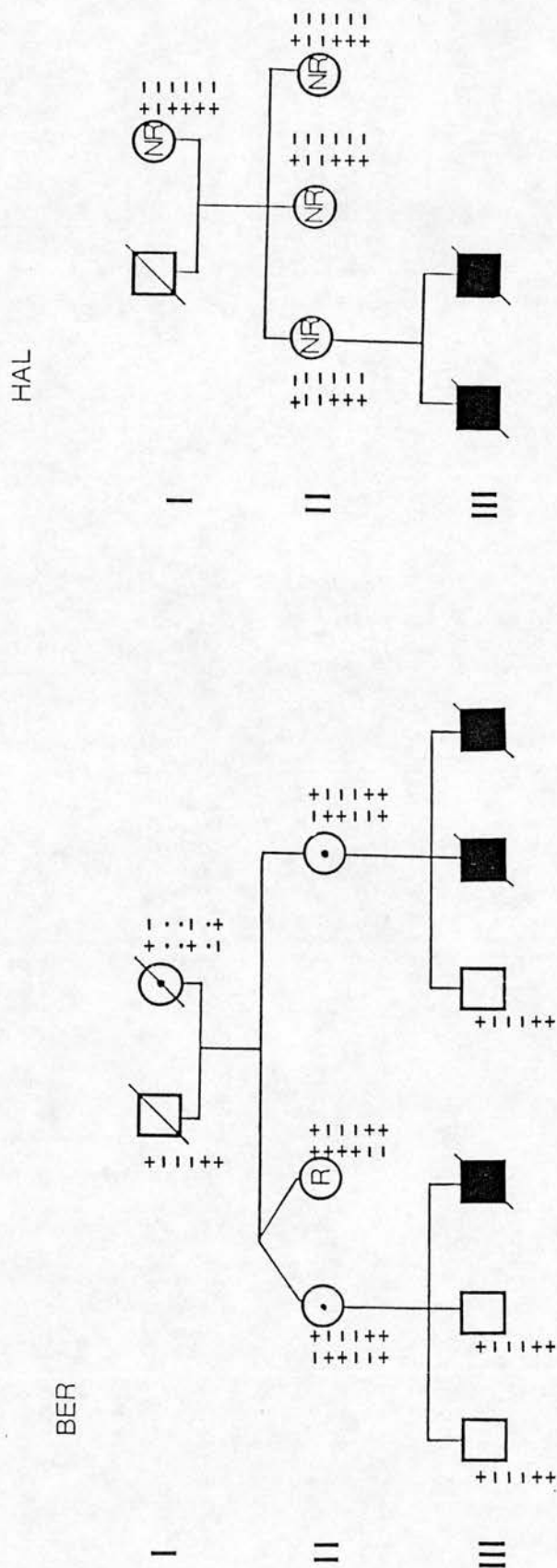


Figure 5.8b Pedigrees with haplotypes of the five families with XSCID. + indicates presence of a polymorphic site and - indicates its absence. The probe order on the pedigrees is cpX203, p8, cpX289, pSPT/PGK, pX65H7, pDP34. ○ obligate carrier female. ○ female with a non-random pattern of X inactivation. ○ female with a random pattern of X inactivation. ○ female of unknown carrier status, homozygous for the BstXI polymorphism detected by pSPT/PGK. • result not obtained. Recombinations are marked by arrows.

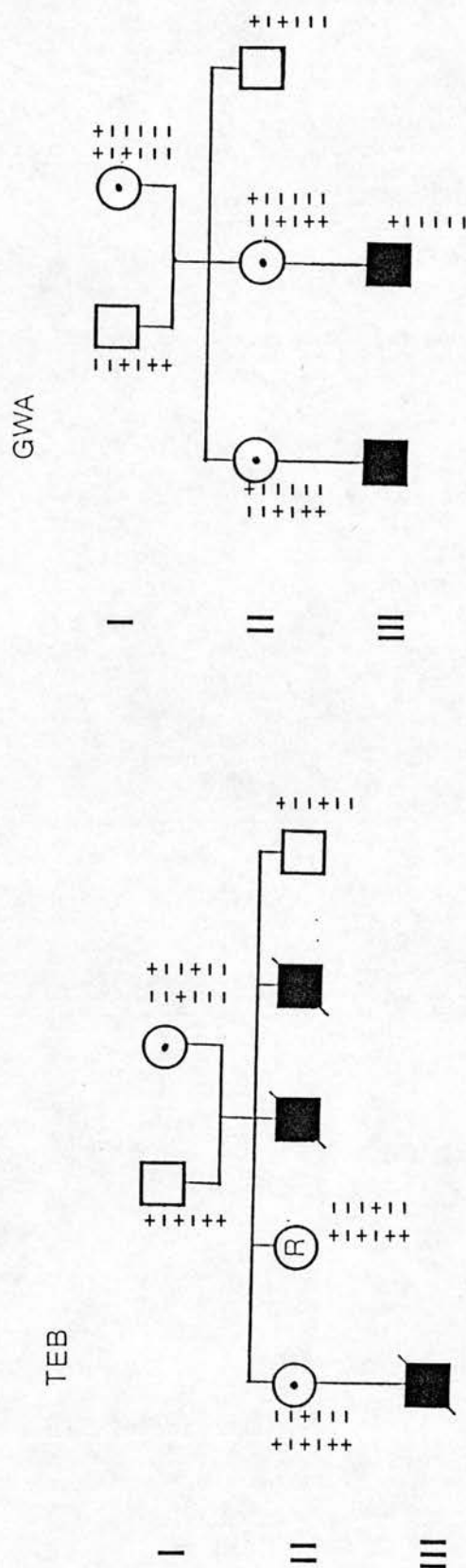


Figure 5.8c Pedigrees with haplotypes of the five families with XSCID. + indicates presence of a polymorphic site and - indicates its absence. The probe order on the pedigrees is cpX203, p8, cpX289, psPT/PGK, pX65H7, pDP34. \odot obligate carrier female. \odot female with a non-random pattern of X inactivation. \odot female with a random pattern of X inactivation. \odot female of unknown carrier status, homozygous for the BstXI polymorphism detected by psPT/PGK. \cdot result not obtained. Recombinations are marked by arrows.

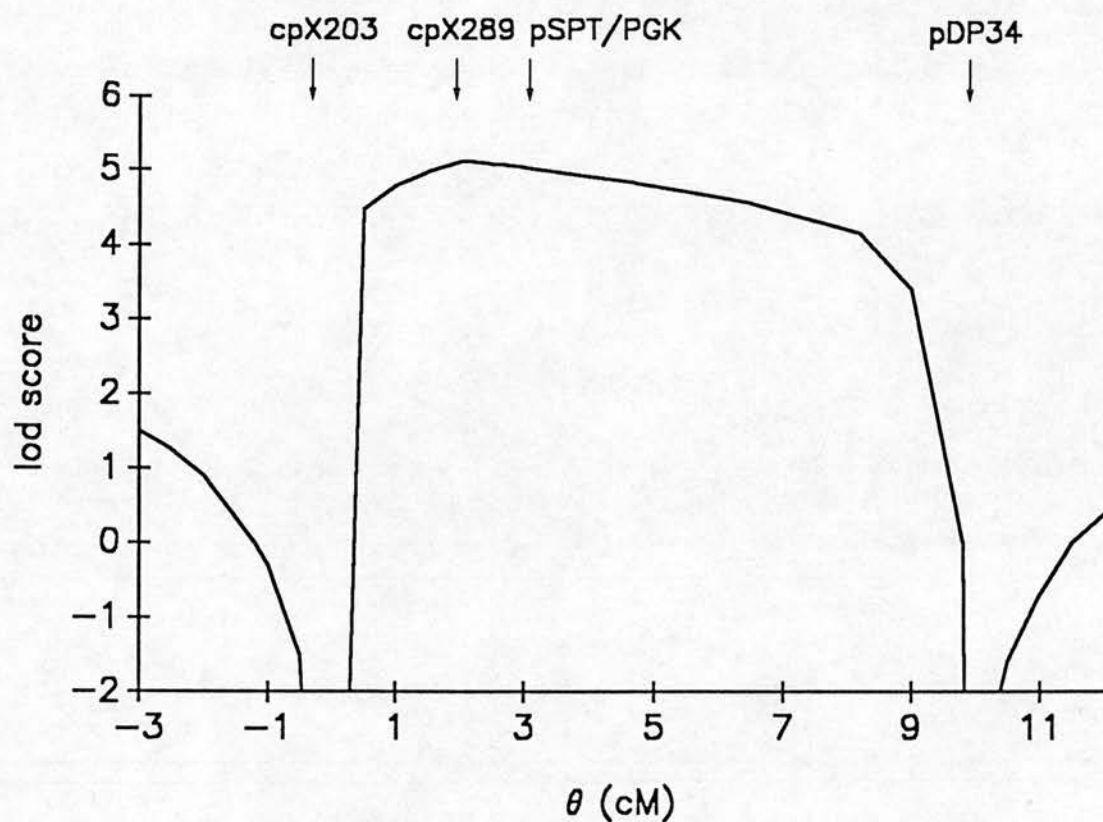


Figure 5.9 Lod scores calculated by LINKMAP analysis for the XSCID locus with cpX203, cpX289, pSPT/PGK and pDP34 as fixed points. A recombination rate of 0.02 was used between cpX203 and cpX289, 0.01 between cpX289 and pSPT/PGK and 0.07 between pSPT/PGK and pDP34.

5.2.4 X-linked Agammaglobulinaemia

Two families, pedigrees CAS and MON, referred for counselling after the initial localisation of XLA to Xq21.3-q22 (Malcolm et al, 1987) were investigated using the probes pXG12, S21, and p212. British families used in the initial study were reinvestigated with the probe p212. The pedigrees and results of families CAS, MON and the families informative for p212 are shown in Appendix 3. Families CAS and MON were informative for S21, with a lod score of 2.4 at $\theta = 0$, but were uninformative for pXG12. Combining the data from these families with other published data the maximum lod score is 12.9 at $\theta = 0$ for S21 and 6.6 at $\theta = 0$ for pXG12. No recombinations were observed between the disease locus and p212 and the maximum lod score was 3.74.

One of the first XLA families to be documented was also investigated (Jamieson and Kerr, 1962). The pedigree is shown in figure 5.10. Subjects IV₄, IV₅ and IV₇ have died since the family was first reported and subjects IV₆, IV₈ and IV₉ have been born since 1962.

Two males, subjects IV₄ and IV₅, had been investigated in detail when the family was first presented. Both had profound hypogammaglobulinaemia and failed to produce any functional antibodies. Subject IV₄ developed a lower motor neuron paralysis affecting the muscles of the right leg and although there was evidence of infection with poliovirus type 2 and type 3 in the family he produced no antibodies to their serotypes. He had a positive Mantoux test indicating that his cell mediated immunity was normal. Both males died in infancy of bacterial infections.

Subject IV₉ has complete absence of circulating B cells in peripheral blood and lacks all immunoglobulin isotypes. His bone marrow has not been investigated for presence or absence of pre B cells. He remains well on intravenous immunoglobulin replacement therapy.

An additional seven males in the pedigree died at less than three years of age. No females died in infancy. The cause of death in II₂ was gastroenteritis, II₅ bronchopneumonia, II₆ miliary tuberculosis and diarrhoea, and in III₅, III₆ and III₉ bronchopneumonia. IV₃ presented at six months of age with diarrhoea and failure to thrive and after he died at eighteen months post mortem examination showed miliary tuberculosis.

The surviving family members who could provide linkage information were traced and samples taken for DNA extraction. DNA was extracted from II₃, II₄, III₄, III₈ and IV₁ in Manchester. Filters of TaqI digested and PstI digested DNA from these individuals were kindly given by Dr A Read. An EBV transformed cell line from IV₉ was generously given by Dr C Roifman (Toronto).

The results obtained for 19.2, pXG12 and S21 are shown on the simplified pedigree in figure 5.11. The family was uninformative for the probe p212. The obligate carrier sisters, III₄ and III₈ inherited different alleles from their mother for the most closely linked probes, pXG12 and S21. Their mother is homozygous for 19.2. III₄ is heterozygous for all three probes and her normal son has inherited the grandmaternal haplotype. III₈ is homozygous for pXG12 and S21. She is heterozygous for 19.2 and her affected son has inherited the grandmaternal allele. Thus the two obligate carrier sisters did not inherit the same maternal X for the region Xq21.3-22 and both the normal and affected males inherited that region of the X chromosome from their grandmother.

Non-paternity or confusion over sample labelling was excluded using the site specific hypervariable probe p λ g3 (Jeffreys et al, 1988).

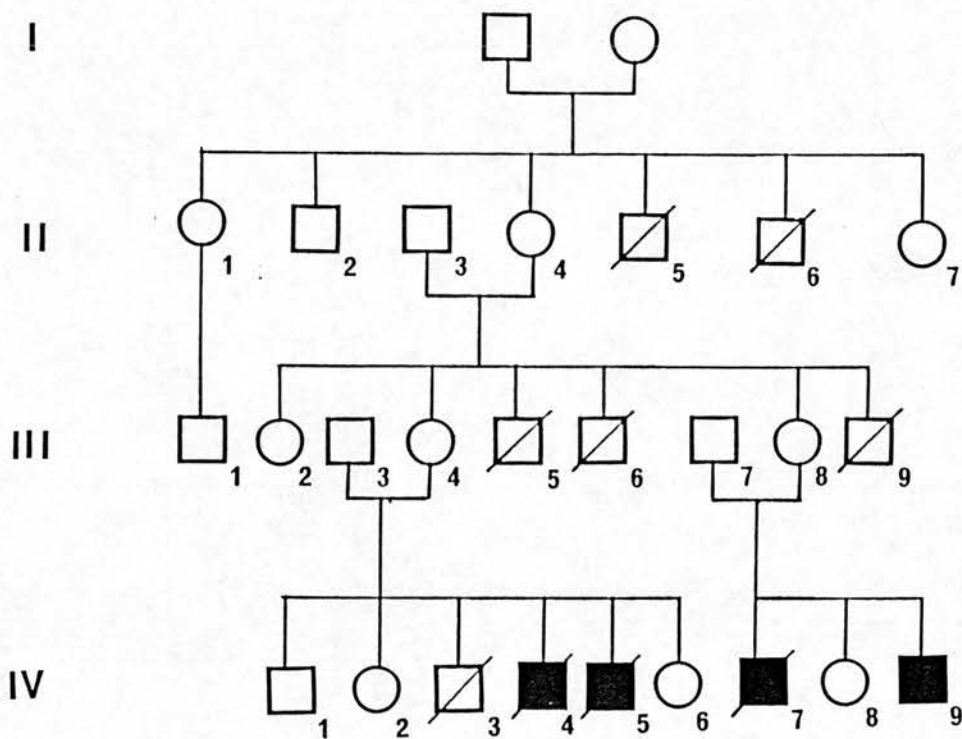


Figure 5.10 Pedigree JAM

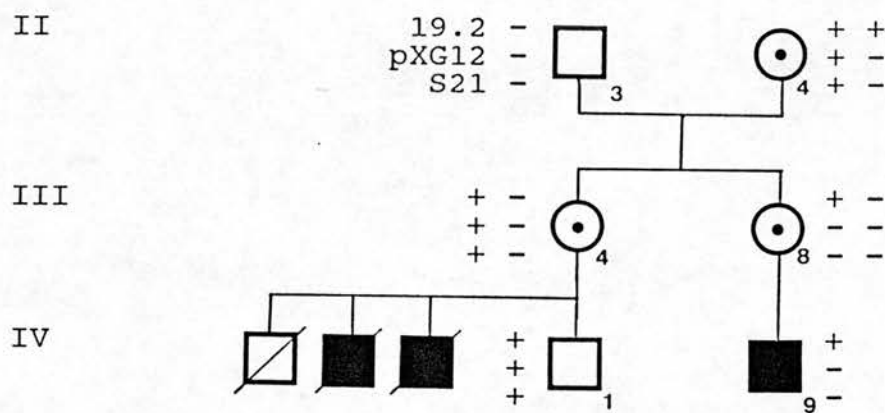


Figure 5.11 Simplified pedigree showing 19.2, pXG12 and S21 results on for individuals in the JAM pedigree from whom DNA was obtained.

The data for 19.2, pXG12 and S21 were analysed using the LINKMAP section of LINKAGE version 4.7 using a distance of 4 cM between 19.2 and pXG12 and 2 cM between pXG12 and S21 (Arveiler et al, 1987). Fifteen points were calculated in each interval and therefore the first figure calculated distal to S21 was for the position 3.3cM from S21. The lod score at this position was -1.8. The maximum lod score between 19.2 and pXG12 was -3.8 and between pXG12 and S21 was -6.5 (figure 5.12). As a lod of -2 is usually accepted as excluding linkage this excludes linkage of the disease to Xq21.3-22 between 19.2 and S21 in this family, but does not exclude linkage 3.3cM distal to S21.

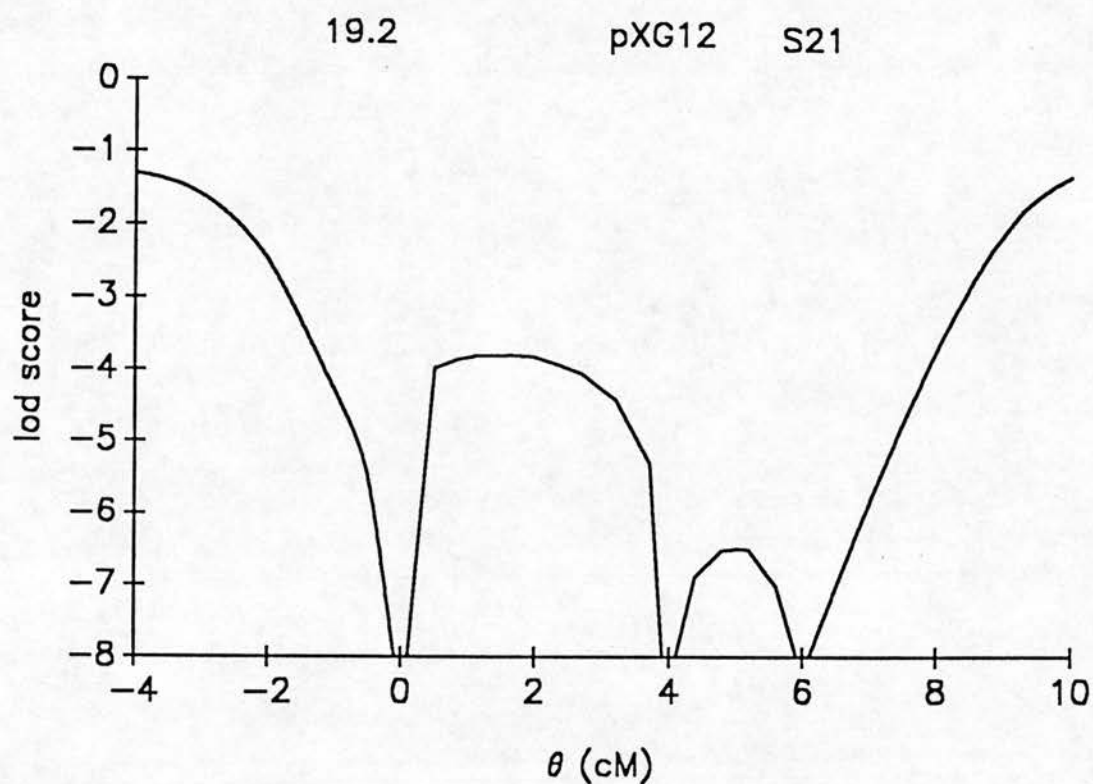


Figure 5.12 LINKMAP analysis of the 19.2, pXG12 and S21 results from pedigree JAM to calculate the probability of the disease locus in this family mapping to this region of the X chromosome.

CHAPTER 6: Results of X Inactivation Studies

6.1 Use of X Inactivation Analysis for Carrier Detection

6.1.1 Information Content of Probes

Thirteen normal females were tested for polymorphisms for pSPT/PGK and three heterozygotes were found. Two out of eighteen normal females were heterozygous for the polymorphism detected by HPRT. Seven obligate carriers of X-linked severe combined immunodeficiency were tested. Two of these were heterozygous for the pSPT/PGK polymorphism and none for the HPRT polymorphism. Twenty five women at risk of being carriers of the disease were tested, nine were heterozygous for pSPT/PGK and two for HPRT.

Combining these figures 8% of women were heterozygous for the HPRT polymorphism and 31% of women were heterozygous for the pSPT/PGK polymorphism. One woman of Asian origin was homozygous for the rare allele of the HPRT polymorphism. The frequencies of the alleles observed for the HPRT polymorphism were 0.94 and 0.06 in the 100 X chromosomes tested and hence the probability of a woman being heterozygous for this polymorphism is 11.3%. Approximately 40% of women will therefore be heterozygous for one or both polymorphisms and can be offered investigation.

6.1.2 Normal Controls

DNA extracted from the E positive cell fraction of two normal females was shown to have a random X inactivation pattern using pSPT/PGK and DNA from E positive cells of one control female was shown to have a random pattern using HPRT (figure 6.1). In addition Fearon has shown a random X inactivation pattern in E positive cells in one female control with pSPT/PGK in one female control with HPRT (Fearon et al, 1987).

6.1.3. Confirmation of Method in X-linked Pedigrees

There were three pedigrees which were clearly X-linked at the start of the study. Two obligate carriers from these families were heterozygous for the pSPT/PGK polymorphism, subject I₂ in pedigree TEB and subject II₂ in pedigree KHA. DNA extracted from the E positive (T lymphocyte enriched) fraction from both of these women showed a non random pattern (figure 6.2). In both of these cases tracking a haplotype of p8 - cpX289 - pSPT/PGK - pX65H7 in the family demonstrated that the mutant gene was on the inactive X chromosome.

The obligate carrier in pedigree TEB was heterozygous for the linked probe cpX289. Her daughter, subject II₄ was at low risk of being a carrier as she had received the same allele as her normal brother and the opposite allele from her carrier sister (fig 5.8). She was heterozygous for the polymorphism detected by pSPT/PGK and

methylation analysis of DNA from E positive cells showed a random X inactivation pattern consistent with these results (fig. 6.2)

The obligate carrier from pedigree KHA was homozygous for cpX289 itself but heterozygous for two probes flanking it. For both of these flanking markers, cpX203 and pX65H7, her daughter, subject III₇, inherited the same allele as her normal brother and the opposite allele from her affected brother and obligate carrier sister (fig 5.8). These results indicated that she was at low risk of being a carrier. Subject III₇ was heterozygous for the polymorphisms detected by pSPT/PGK and methylation analysis demonstrated a random X inactivation pattern in the E positive cell fraction (fig 6.2).

Pedigree BER (fig 5.8) is the third family with a clearly X-linked pedigree. The haplotypes for the obligate carrier have been inferred. The two obligate carrier sisters inherited the opposite alleles from their at risk sister, subject II₃, for the flanking probes cpX203 and pDP34. Subject II₃ was therefore at low risk of being a carrier of the disorder. She was heterozygous for the pSPT/PGK polymorphism and had a random pattern of X inactivation in the T cell enriched DNA.

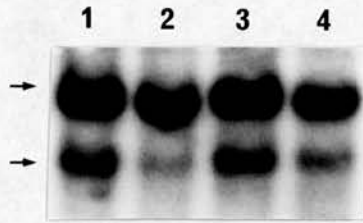


Figure 6.1a Analysis of X Inactivation in E positive Cells from Two Control Subjects using the Polymorphism Detected by pSPT/PGK.

In the first and third tracks the DNA has been digested with PstI and BstXI. In the second and fourth tracks the DNA has been digested with PstI/BstXI and HpaII. The 1.05 and 0.9kb polymorphic bands are indicated by arrows. Both polymorphic bands are seen in all tracks.

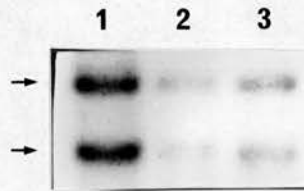


Figure 6.1b Analysis of X Inactivation in E Positive Cells from One Control Subject using the Polymorphism Detected by HPRT.

The DNA has been digested with BamHI/PvuII in the first track, BamHI/PvuII and HhaI in the second track and BamHI/PvuII and HpaII in the third track. The filter has been hybridised with HPRT-600. The 18 and 12kb polymorphic bands are indicated by arrows. Both polymorphic bands are of equal intensity in all tracks.

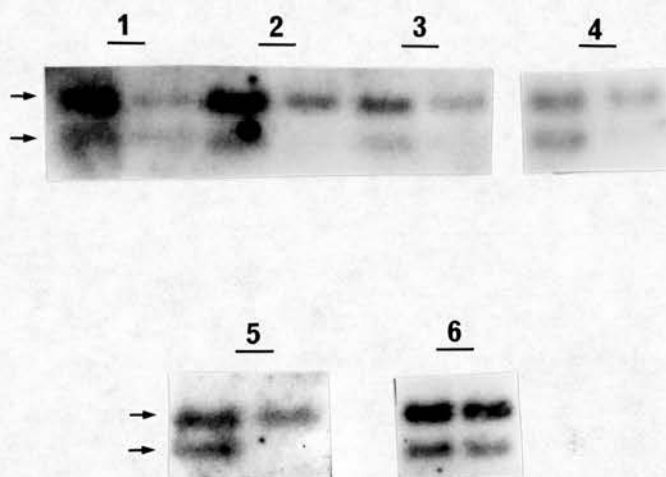


Figure 6.2 Analysis of X Inactivation Patterns

Paired tracks have been run from each DNA sample. In the first track from each sample the DNA has been digested with PstI and BstXI. In the second track from each sample the DNA has been digested with PstI/BstXI and HpaII. The filters have been hybridised with pSPT/PGK. The polymorphic bands are indicated by the arrows. Sample 1 is E positive cell DNA from subject III₂ in pedigree KHA. Sample 2 is E positive cell DNA, sample 3 is E negative cell DNA and sample 4 is DNA from an EBV transformed cell line from the obligate carrier, subject II₂, in pedigree KHA. Sample 5 is E positive cell DNA from the obligate carrier, subject I₂, in pedigree TEB. Sample 6 is E positive DNA from subject II₄ in pedigree TEB. In both obligate carriers the lower allele is absent after the addition of HpaII. In the E negative cell DNA from the obligate carrier in pedigree KHA the lower allele is absent after the addition of HpaII. Both polymorphic bands were present after the addition of HpaII in the samples from their daughters who were predicted to be at low risk of being carriers using linked DNA probes.

6.1.4. Testing for X-linkage

The proband in pedigree HAL is clearly a carrier of severe combined immunodeficiency as she has had two affected sons, however the disease could be autosomal recessive or X-linked recessive (figure 6.3a). Her mother had two brothers who died in infancy which is suggestive of X-linked inheritance but no details were available. The proband, her mother and both sisters were informative for the pSPT/PGK polymorphism. The proband was found to have a non random pattern of X inactivation in DNA extracted from the E positive cell fraction, losing the 1.05kb band after the addition of HpaII (figure 6.3b). Her mother and both sisters also had a non random pattern of X inactivation in T cells, all losing the 1.05kb band after the addition of HpaII.

Pedigree MOR (figure 6.4a) has a similar structure to pedigree HAL. The proband had an affected male child who died in spite of bone marrow transplantation. A fetal blood sample was taken for prenatal diagnosis in her second pregnancy. There were no T lymphocytes and the PHA response was absent. The affected male fetus was aborted. Prenatal diagnosis was also carried out in the third pregnancy which resulted in a normal male child. Having had two affected males diagnosed this woman was either a carrier of autosomal recessive or X-linked recessive SCID. She was heterozygous for the pSPT/PGK polymorphism. Methylation analysis of DNA from the E positive cells showed a random pattern of X inactivation (figure 6.4b) suggesting that in this family the disease is autosomal recessive.

The proband in pedigree LAW has had only one child, a male with SCID. Adenosine deaminase deficiency was excluded as the cause of the disease. The affected child received a mismatched bone marrow transplant but died. The proband has one normal brother and there is no family history of males dying in infancy.

The proband was heterozygous for pSPT/PGK. Methylation analysis showed a non random pattern of X inactivation and she is therefore a carrier of the X-linked form of the disease.

The allele that is lost after the addition of HpaII represents the active X chromosome and the allele that remains represents the inactive X chromosome. In this case the band remaining, which represents the X chromosome carrying the disease mutation, is the allele inherited from the mother (figure 6.5).

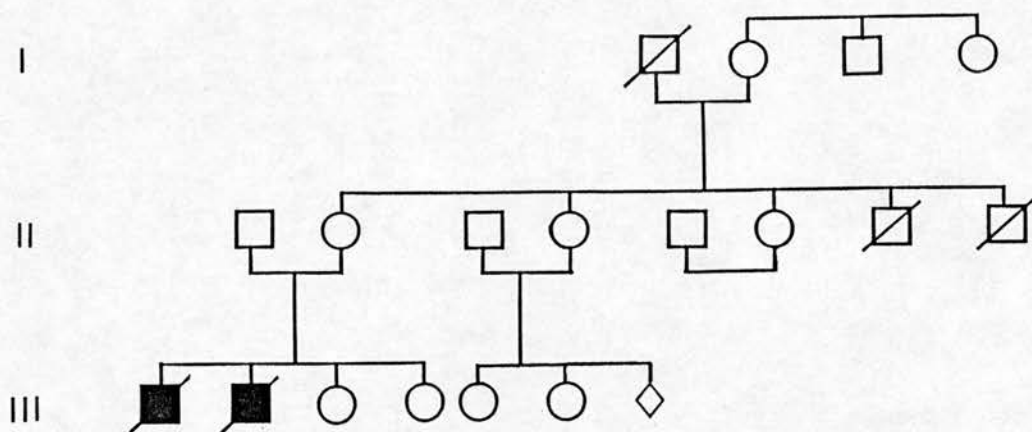


Figure 6.3a Pedigree HAL

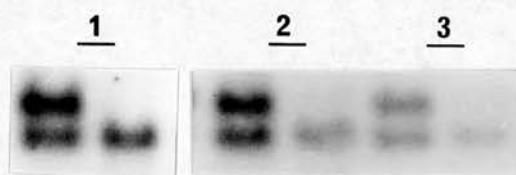


Figure 6.3b Analysis of X Inactivation Patterns in Pedigree HAL

Paired tracks have been run from each DNA sample. In the first track the DNA has been digested with PstI/BstXI and in the second track of each sample the DNA has been digested with PstI/BstXI and HpaII. The filter has been hybridised with pSPT/PGK. Sample 1 is E positive cell DNA from subject II₂, sample 2 is E negative cell DNA from subject II₄ and sample 3 is granulocyte DNA from subject II₄. In all three samples the 1.05kb band is absent after the addition of HpaII.

Figure 6.3c

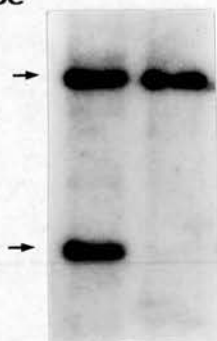


Figure 6.3d

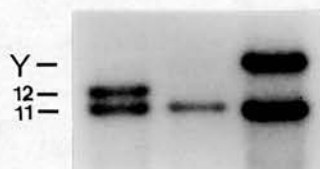


Figure 6.3c pSPT/PGK bands in a BglI digest of DNA from subject II₄ in the first track and from the chorion sample in the second track. Arrows indicate the 5 and 12 kb bands of the polymorphism. The fetus has inherited the upper allele, which represents the X chromosome carrying the normal gene.

Figure 6.3d pDP34 hybridised to TaqI digested DNA from a female control, the chorion sample and a male control. The arrow indicates the Y specific band. The 11 and 12 kb bands show the X chromosome polymorphism detected by pDP34. The fetus does not have a Y specific band.

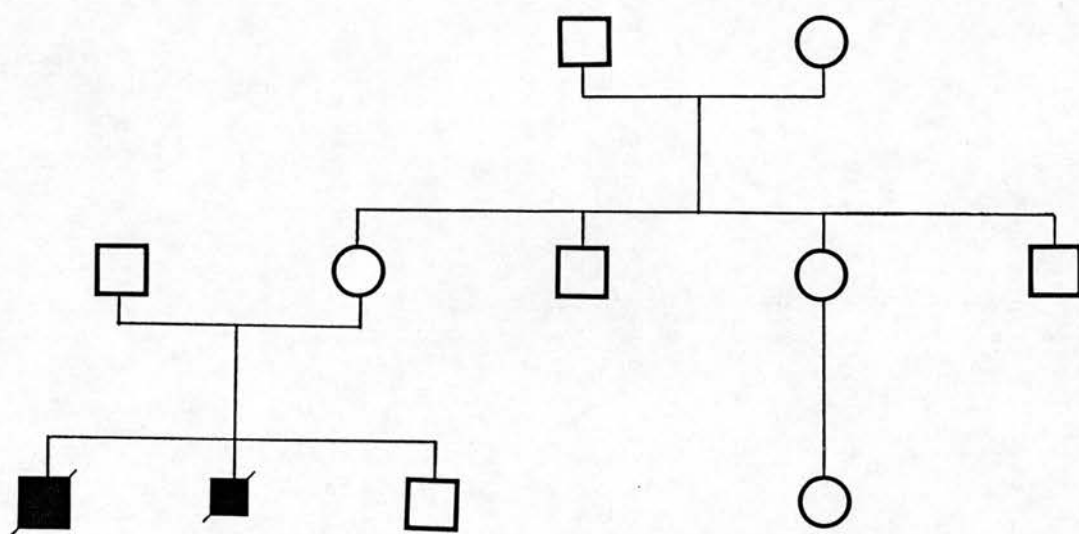


Figure 6.4a Pedigree MOR



Figure 6.4b X Inactivation Analysis of E Positive DNA from the Proband in Pedigree MOR.

The DNA in the first track has been digested with PstI/BstXI and the DNA in the second track has been digested with PstI/BstXI and HpaII. The filter was hybridised with pSPT/PGK. Both bands remain after the addition of HpaII.

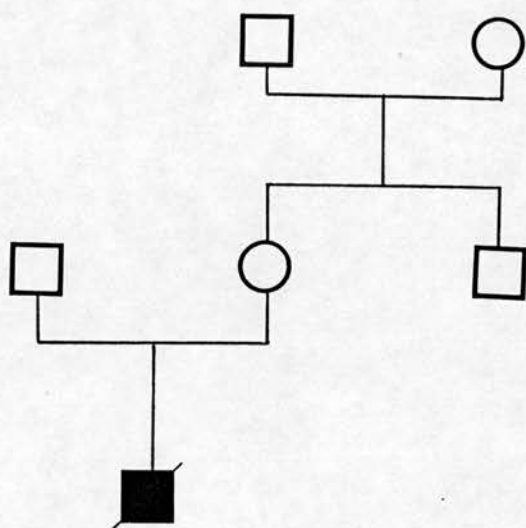


Figure 6.5a Pedigree LAW

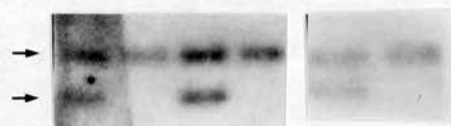


Figure 6.5b X Inactivation Analysis. The three DNA samples are from E positive cells, E negative cells and granulocytes from the proband in Pedigree LAW. The DNA in the first track from each sample has been digested with PstI/BstXI and the DNA in the second track has been digested with PstI/BstXI and HpaII. The filter was hybridised with pSPT/PGK. The 1.05 and 0.9kb polymorphic bands are indicated by arrows. The 0.9kb allele is absent in the E positive, E negative and granulocyte track after the addition of HpaII.

The proband in pedigree EIS has one normal daughter and one son who had severe combined immunodeficiency. He died three weeks after his first admission to hospital at the age of seventeen months. The proband has two normal brothers and there was no previous family history of males dying in infancy.

The proband was heterozygous for the HPRT polymorphism. After the addition of HhaI the 18kb band is fainter than the 12kb band and after the addition of HpaII the 12kb band is lost when using HPRT-600 as the probe (figure 6.6). Using this probe in a sample with non random X inactivation the band representing the inactive X chromosome becomes fainter after the addition of HhaI and the band representing the active X chromosome is lost after the addition of HpaII. This is consistent with the proband being a carrier of X-linked disease and with the 18kb allele representing the inactive X chromosome which carries the disease mutation.

The proband's mother was heterozygous for the HPRT polymorphism and her father had the 18kb band. Therefore the band representing the inactive X chromosome in the proband has been inherited from her father. Paternity was checked using two locus specific probes, M27 β and p λ g3, because if this had been a case of non-paternity the subject could have inherited this allele from her heterozygous mother. Clarifying this has important implications for the counselling of the extended family.

The proband in this family is a carrier of the disease as the result of a new mutation inherited from her father.

The couple in pedigree SPE have had one daughter and an affected son who died in spite of bone marrow transplantation. The proband was heterozygous for the HPRT polymorphism. Methylation analysis of DNA extracted from the E positive cell fraction showed the normal random pattern (figure 6.7). She is therefore not a carrier of the X-linked form of the disease. Her son could either have carried a new mutation in the X-linked gene or had autosomal recessive SCID.

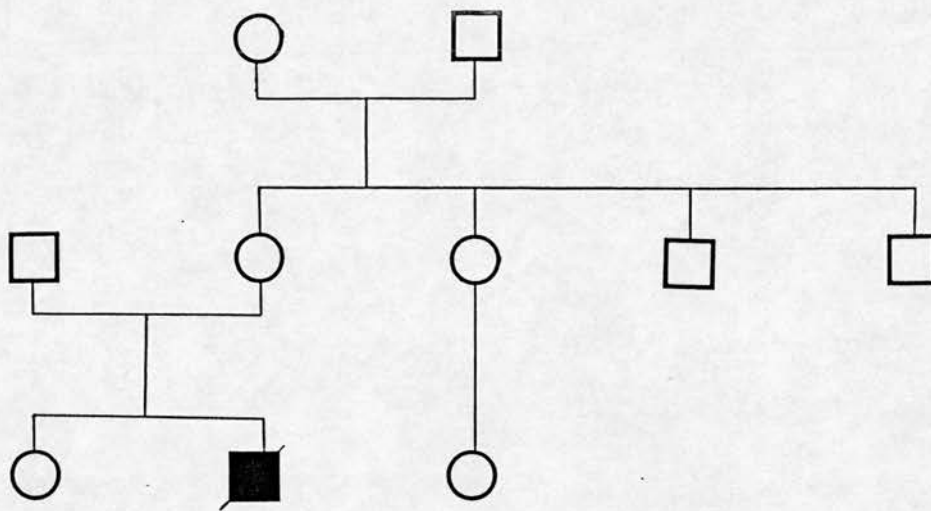


Figure 6.6a Pedigree EIS

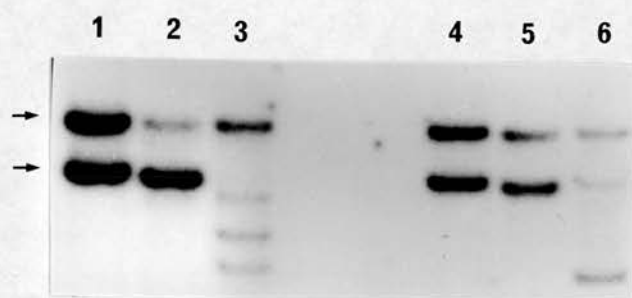


Figure 6.6b X Inactivation Analysis using HPRT

Tracks 1, 2, and 3 contain E positive cell DNA and tracks 4, 5 and 6 contain granulocyte DNA from the proband in Pedigree EIS. The DNA in tracks 1 and 4 has been digested with BamHI and PvuII. The DNA in tracks 2 and 5 has been digested with BamHI/PvuII and HhaI. The DNA in tracks 3 and 6 has been digested with BamHI/PvuII and HpaII. The filter was hybridised with HPRT-600. Arrows indicate the 18 and 12kb polymorphic bands. In the BamHI/PvuII/HhaI tracks the 18kb band is fainter than the 12kb band in E positive DNA (track 2) but not in granulocyte DNA (track 5). In the BamHI/PvuII/HpaII tracks the 12kb band is absent in the E positive DNA (track 3) but is present, and of equal intensity with the 18kb band, in the granulocyte DNA (track 6). The decreased intensity in the 18kb band in the HhaI track and the absence of the 12kb band in the HpaII track of the E positive cell DNA shows non-random use of the X chromosome in these cells. As the band representing the active X chromosome is digested into smaller fragments after the addition of HpaII but the band representing the inactive X chromosome is digested into smaller fragments after the addition of HhaI in approximately 90% of cases the 12kb represents the active X chromosome. The presence of bands of equal intensity after the addition of methylation sensitive endonucleases in the granulocyte DNA tracks indicates random use of the X chromosome in these cells.



Figure 6.6c X Inactivation Analysis of EBV Transformed Cell Lines.

The DNA in the first track has been digested with BamHI and PvuII to show the position of the polymorphic bands. The DNA in the remaining five tracks is from five EBV lines from the proband in Pedigree EIS and has been digested with BamHI/PvuII/HpaII and hybridised with HPRT-600. The two bands are of equal intensity in DNA from the first EBV line. In the remaining four EBV lines the 12kb band is absent.

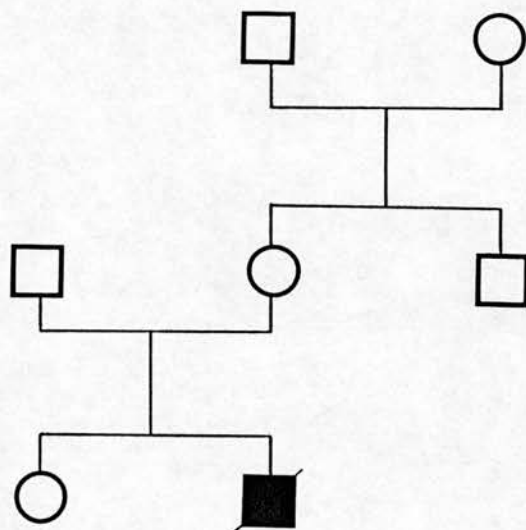


Figure 6.7a Pedigree SPE

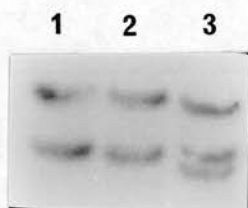


Figure 6.7b X Inactivation Analysis of E Positive Cells from the Proband in Pedigree SPE using HPRT.

The DNA in the first track has been digested with BamHI and PvuII. The DNA in the second track has been digested with BamHI/PvuII and HhaI and the DNA in the third track has been digested with BamHI/PvuII and HpaII. The filter has been hybridised with HPRT-600. The presence of bands of equal intensity after the addition of both methylation sensitive endonucleases shows random X-chromosome usage in these cells.

6.1.5 Use of PGK for Carrier Detection and as a Linked Probe

It has been shown in section 5.2.3 that the probe pSPT/PGK, which detects non-random X chromosome usage in heterozygous female carriers of XSCID, is also linked to the XSCID disease locus. When methylation analysis reveals a non-random pattern of X chromosome usage the allele that remains after the addition of HpaII represents the inactive X and hence the X chromosome carrying the defective gene.

In family HAL there are no males, normal or affected, who could be used to assign phase. The three sisters are homozygous for the linked probe cpX289. All four women are heterozygous for pSPT/PGK and in all four the pSPT/PGK allele which remained after the addition of HpaII was the 0.9kb band. The X chromosome carrying the defective gene in these females is therefore the chromosome which has the BstXI polymorphic site present.

During the time that the family was being studied the second sister, II₄, became pregnant for the third time. The couple were told that she was a carrier of the disease and they requested prenatal diagnosis. As she was homozygous for the polymorphism detected by cpX289 this could not be used for prenatal diagnosis. They were offered the following options: ultrasound sexing at eighteen weeks gestation followed by fetal blood sampling in a male fetus, fetal sexing on chorion villus sampling with termination of all males, or DNA studies on chorion villus sampling to determine sex and also high or low risk depending on the pSPT/PGK allele inherited. The couple chose to have chorion sampling for determination of sex and DNA analysis with pSPT/PGK, deciding to terminate a male fetus who had inherited the allele carrying the high risk and to continue the pregnancy if a male fetus had inherited the low risk allele but to confirm this result on fetal blood sampling.

The results of the DNA analysis are shown in figure 6.3. The BstXI and BglI polymorphisms are in linkage disequilibrium. DNA digested with BglI was probed with pSPT/PGK and DNA digested with TaqI was probed with pDP34 which detects a Y specific band. The fetus was shown to be female using pDP34 and had inherited the low risk allele.

The proband in family LAW has been shown to be a carrier by methylation analysis using pSPT/PGK (figure 6.5). The allele remaining after the addition of HpaII was the 1.05kb band and the 0.9kb band is no longer seen on the autoradiograph. The chromosome with the polymorphic site therefore represents the normal X and the chromosome that does not have the polymorphic site carries the defective gene. A male fetus inheriting the pSPT/PGK lower allele when the DNA is digested with BstXI or BglI has a low probability of being affected and a male fetus inheriting the upper allele has a high risk of being affected.

6.2 Use of X Inactivation to Investigate the Expression of the Gene Defective in XSCID

Gene expression was studied in the E positive and E negative cell fractions, granulocytes and EBV transformed cell lines. Seven of the eight women above who were found to have non-random X chromosome usage in their E positive cells were included in the study though results were not obtained for all cell types on each woman. The results are shown in Table 6.1.

Subject	Pedigree	Epos	Eneg	Adherent	Granulocytes
1	KHA	N	N	-	R
2	LAW	N	N	N	N
3	EIS	N	-	-	R
4	HAL	N	N	-	N
5	HAL	N	-	-	R
6	HAL	N	-	-	R

Table 6.1 Results of methylation analysis in E positive, E negative, adherent cells and granulocytes in six subjects. N indicates non-random use of the X chromosome. R indicates random use of the X chromosome. When no result was obtained the symbol - is used.

6.2.1 Gene Expression in T Lymphocytes

The T lymphocyte enriched (E positive) fraction contained on average 84% (range 74-93%) CD3 positive cells. In addition there were 7-23% HNK1 positive cells present. There was less than 1% B lymphocyte (CD20) and monocyte (CD14) contamination in this fraction. As shown above a non-random pattern was found in this cell fraction in all of the carriers.

6.2.2. Gene Expression in E Negative Cells

The E negative fraction was shown to contain predominantly monocytes, B lymphocytes and NK cells by specific monoclonal antibody staining, but T lymphocyte contamination occurred in some preparations. A result for this cell fraction was obtained in subjects 1, 2 and 4. A non-random pattern of X inactivation was found in all three women. In addition a monocyte enriched fraction was kindly separated by Dr R.Callard for subject 2 by plastic adherence. In this population 46% of the cells were CD14 positive, 17% CD3 positive and 11% CD20 positive. This monocyte enriched fraction also showed a non-random pattern of X inactivation.

6.2.3 Gene Expression in Granulocytes

The granulocyte fraction was 99% pure both by morphological criteria and by immunofluorescence with the CD15 antibody. Variation was found in the X inactivation patterns of the six subjects for whom

results were obtained. Subjects 2 and 4 had a non-random pattern but in subjects 1, 3, 5 and 6 two bands remained after HpaII digestion. Not only was there variation between unrelated carriers but also within a family. Subject 4 had a non-random pattern of X inactivation in her granulocytes but her mother and sister, subjects 5 and 6, were found to have a random pattern of X chromosome usage in their granulocytes.

6.2.4 Gene Expression in B Lymphocytes

As it was impossible to isolate sufficient B lymphocytes for DNA extraction from the blood sample taken, multiple EBV transformed lines were established from each subject. The lines were grown only until sufficient cells (5×10^6) were available for DNA extraction to minimise the possibility of the lines becoming oligoclonal. The X inactivation pattern was investigated in individual lines. The number of lines examined and the proportion showing non-random X chromosome usage is shown in Table 6.2. In subject 2, ten individual lines were tested and a non-random pattern found in each. The same X chromosome was active in each of the ten EBV lines and this corresponded to the active X in the woman's T lymphocytes. These results provide good evidence that the defect causing XSCID is expressed in this woman's B lymphocytes. In contrast, in subjects 3, 5 and 6 whilst some EBV transformed B lymphocyte lines had become non-random others still retained random use of the X-chromosome. No individual was found who showed random X chromosome usage in all EBV transformed cell lines and in each EBV line which became non-random the active X corresponded to the active X in that woman's T lymphocytes.

Subject	EBV Lines
	Non-Random
1	1/1
2	10/10
3	4/5
4	-
5	1/6
6	3/8

Table 6.2 Results of methylation analysis from individual EBV transformed cell lines from six subjects. The number of lines with non-random X chromosome usage is shown in the numerator and the number of cell lines tested from each individual in the denominator. No results were obtained from subject 4.

6.2.5 Gene Expression in Total Circulating Haematopoietic and Lymphoid Cells

Analysis of DNA made from whole blood from subject 2 showed a non-random pattern of X inactivation, as had her E positive, E negative, monocyte and granulocyte preparation. Analysis of DNA prepared from whole blood from the obligate carrier, II₄, in pedigree TEB also showed a non-random pattern of X inactivation. In this subject the only other cell fraction investigated was the E positive fraction which also showed loss of the lower band after the addition of HpaII. In the remaining subjects the band representing the X carrying the normal gene was fainter than the band representing the X carrying the defective gene after the addition of HpaII but both bands were still present (figure 6.8). X inactivation analysis of whole blood would not be reliable for carrier testing.

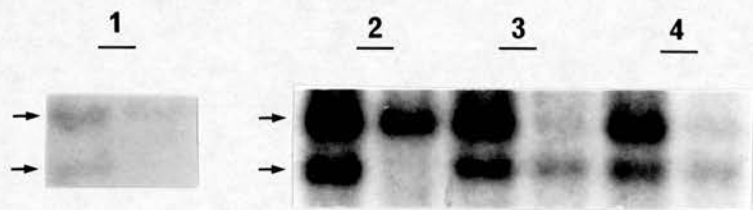


Figure 6.8 X Inactivation Analysis of DNA Extracted from Venous Blood of Carriers of XSCID

The four DNA samples have been extracted from whole blood. The DNA in the first track from each sample has been digested with PstI/BstXI and the DNA in the second track with PstI/BstXI and HpaII. The filter was hybridised with pSPT/PGK. The 1.05 and 0.9kb polymorphic bands are indicated by arrows. The first sample is from subject II₄ in pedigree TEB. The second sample corresponds to subject 2, the third to subject 4 and the fourth to subject 5 in Tables 6.1 and 6.2.

CHAPTER 7. Discussion: Mapping

7.1 Genetic Map of Proximal Xq

7.1.1 Order of Probes used in this Study

The investigation of both recombinant chromosomes and the X chromosome deletion in patient MB have provided information about the order of probes on proximal Xq.

From the recombinant chromosome data cpX289, cpX203 and pX65H7 are proximal to the XY homologous region along with M27 β , pTAK8, p8, pSPT/PGK. Within this group order information was only obtained from one recombinant chromosome. This placed cpX203 proximal to p8 and pSPT/PGK but was uninformative for M27 β , pTAK8, cpX289 and pX65H7. No information was obtained about the relative order of M27 β , pTAK8, p8, cpX289, pSPT/PGK and pX65H7 or about the order of M27 β , pTAK8 and cpX203 as there were no recombinations between them.

The recombinant chromosome data shows that the probes pXG12 and p212 are distal to the XY homologous region along with 19.2 and S21. One recombinant chromosome gave information about the position of p212 within the group suggesting a localisation distal to 19.2 which was confirmed by the observation that p212 hybridised to DNA from MB. Two recombinant chromosomes were informative for S21 but gave conflicting information about its position relative to 19.2. This was resolved by the MB deletion. S21 hybridised to DNA from MB and is, therefore, distal to 19.2. pXG12 also hybridised to DNA from patient MB and is therefore distal to 19.2.

Results presented here show that G3-1 and pA13.R1 detect the same polymorphism in contrast to their different regional assignments at HGM8 when pA13.R1 was assigned to Xq21-q24 and G3-1 to Xq11-22. Recombinant chromosome 3 in figure 4 gives evidence for their localisation distal to pX65H7. Recombinant chromosome 4 has only one recombination if they are proximal to 19.2 but a double recombination if they are distal to it. Explaining the data with the minimum number of recombinations would, therefore, place G3-1 and pA13.R1 between pX65H7 and 19.2. As they hybridised to DNA from patient MB the region between pDP34 and 19.2 is excluded suggesting a localisation between pX65H7 and pDP34.

The order based on the results in this thesis is (pTAK8 - M27 β - p8 - pSPT/PGK - cpX289 - pX65H7) - (pA13.R1 - G3-1) - pDP34 - 19.2 - (pXG12 - S21 - p212) with cpX203 lying proximal to p8 and pSPT/PGK.

Studies using somatic cell hybrids from females with X;autosome translocations and EBV transformed cell lines from males with X chromosome deletions are in agreement with these findings for all but the localisation of G3-1 and pA13.R1. The results of this study and other published work are discussed below. Combining all the

information the order of the probes is (pTAK8 - M27 β) - cpX203 - p8 - cpX289 - PGK - pX65H7 - pDP34 - 19.2 - (pXG12 - p212) - S21. The position of G3-1 and pA13.R1 in this framework is not clear.

The results in the thesis place cpX203 proximal to p8 and pSPT/PGK but give no information about its position relative to the other probes in this group. Recent studies have shown pTAK8 and M27 β present in a somatic cell hybrid (MOCH) cultured in 6-thioguanine medium and derived from a female with incontinentia pigmenti and an X;9 translocation with the X chromosome breakpoint p11.21 (Sefiani et al, 1988). cpX203 is present in the hybrids grown by HAT selection. As the translocation product with the long arm of the X chromosome active is present in hybrids grown in HAT medium but not in hybrids grown in medium containing 6-thioguanine pTAK8 and M27 β are distal to Xp11 and cpX203 is proximal to Xp11. It is not clear from this data whether cpX203 is on the short arm of the X chromosome or the long arm.

Five cell lines (AnLy, GM73, W4-1A, CerS and CerH,) have given information about the order of p8, pSPT/PGK and cpX289. The information from these cell lines is summarised in figure 7.1.

The AnLy cell line was derived from a female with X linked hypohydrotic ectodermal dysplasia who had an X;9 translocation (Cohen et al, 1972) with the X chromosome breakpoint at Xq13.1 (Zonana et al, 1988b). Hybrids made by HAT selection, and hence retaining the translocation product with the long arm of the X chromosome, have been shown to express the enzyme PGK by starch gel electrophoresis. Cell lines grown in 8-azaguanine lose the chromosome expressing HPRT and also lose PGK expression (Shows and Brown, 1974). The probes p8 and cpX289 do not hybridise to DNA made from the AnLy cell line which contains Xq13.1-qter (Arveiler et al, 1987). These probes therefore lie centromeric to the breakpoint and PGK distal to the breakpoint Xq13.1. The cell line W4-1A containing Xq13-Xqter is also positive for PGK but negative for cpX289 (Mahtani and Willard, 1988) and the cell line GM73 which contains Xq13-Xqter is positive for PGK but negative for p8 and cpX289 (Arveiler et al, 1987) adding further to the evidence that p8 and cpX289 are proximal to PGK.

The order of p8 and cpX289 depends on information from two somatic cell hybrids CerS and CerH which contain complementary chromosomes of a reciprocal translocation X;22 (q11.2,q13) (Hors-Cayla et al, 1981). CerH contains Xq11.2-qter and expresses PGK while CerS which contains Xpter-Xq11.2 does not express PGK. cpX289 hybridises to CerH but not CerS placing it distal to Xq11.2. However p8 hybridises to both of the cell lines (Arveiler et al, 1987). In addition two probes from the short arm of the X chromosome hybridise to DNA from the CerH hybrid implying that CerH has additional X chromosome DNA to Xq11.2-qter. The results from CerS imply that p8 is proximal to the breakpoint and cpX289 is distal to the breakpoint.

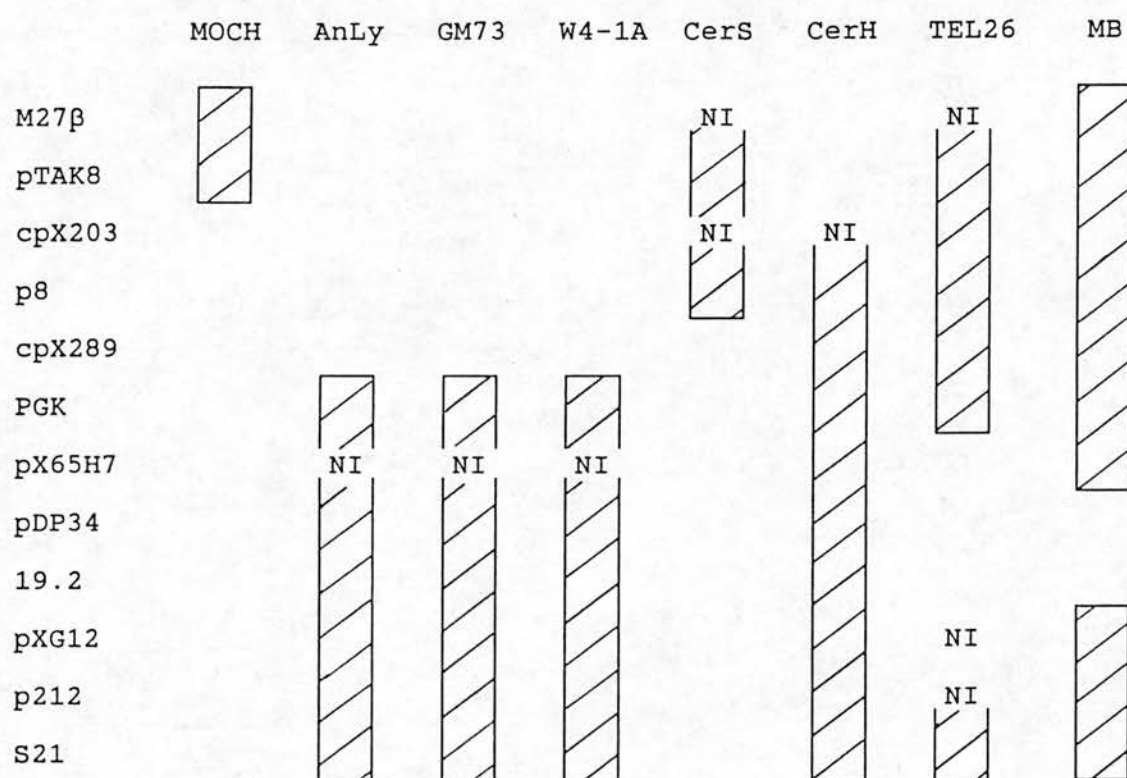


Figure 7.1 Physical mapping of X-linked probes by hybridisation to human-rodent cell hybrids and DNA from males with X chromosome deletions.

Information about the position of pX65H7 has been derived from the cell line (TEL26). This cell line was made from a male with cleft lip and palate, agenesis of the corpus callosum and severe mental retardation who had an X chromosome deletion with breakpoints at Xq13 and Xq21.3 (Tabor et al, 1983). pX65H7 does not hybridise to DNA made from this line (Schwartz et al, 1988). As p8, cpX289 and PGK all hybridise to TEL 26 (Ivens et al, 1988) pX65H7 is distal to PGK.

Distal to the XY homologous region is the group of probes which includes 19.2, pXG12, S21, and p212. Several studies confirm the finding that 19.2 is proximal to S21, pXG12 and p212. Analysis of TEL26 has shown that 19.2 is proximal to S21 (Schwartz et al, 1988) and recombinations in family studies have shown that 19.2 is proximal to pXG12 and p212 (Arveiler et al, 1987). Recombinations in Arveiler's study also showed that both pXG12 and p212 are proximal to S21. There is no evidence for the order of pXG12 and p212.

The original localisations given for G3-1 and pA13.R1 were Xq11-q21 and Xq21-24 respectively (Upadhyaya et al, 1986). Combining the data from recombinant chromosomes and the MB deletion suggests a localisation between pX65H7 and pDP34. However, Schwartz presented deletion mapping results at the Ninth Human Gene Mapping Meeting placing pA13.R1 distal to 19.2 (Schwartz et al, 1987). No information has been published about the position of G3-1 but as shown in this study these probes detect the same polymorphic site. Unfortunately Arveiler did not use either of these probes in his study which was confined to probes detecting PstI or TaqI polymorphisms. There is more evidence for a location distal to 19.2 from deletion mapping than the localisation proximal to pDP34.

The localisation of G3-1 in this study hinges on one recombinant chromosome. Accepting a localisation distal to 19.2 means that a double recombination has occurred on this chromosome and shows that the use of haplotypes on individual recombinant chromosomes can lead to errors. In this study double recombinations occurred on two out of ten recombinant chromosomes. One of the double recombinations occurred between pDP34 and G3-1 and the genetic distance between these markers is not known. The second occurred between pX65H7 and S21 which, based on the results of Arveiler, are more than 10 cM apart (Arveiler et al, 1987). Whilst analysis of recombinant chromosomes can lead to errors it is the only method of probe ordering that does not require cytogenetic expertise and in this panel of probes provides the only evidence for the order of pXG12 - S21 and p212 - S21.

Somatic cell hybrids are an important tool in the localisation of probes. Nearly all of the information about the order of M27 β , pTAK8, cpX203, p8, cpX289 and the locus PGK1 is based on the study of somatic cell hybrids. However the facilities for tissue culture are expensive and considerable expertise is required for the constant checking of the hybrids by karyotyping. There is the potential problem of further rearrangements occurring in culture as demonstrated by the CerS and CerH lines discussed above. The use of somatic cell hybrids, therefore, is expensive, requires more skill and errors can still occur.

Deletion mapping in males with X chromosome deletions is relatively straightforward after the first detailed karyotype has been done. The only potential drawback is the possibility of a more complex rearrangement that cannot be seen using cytogenetic techniques.

All three approaches have given information about the order of some of the probes in this panel that was not given by the other two methods and, therefore, all three have a role. Ideally the order should be based on more than one piece of evidence for each probe and any localisation based on a single piece of evidence should be regarded as tentative until further supporting evidence is found. cpX289 was found to be proximal to PGK1 in four somatic cell hybrids providing strong evidence for the order of these two probes. Similarly the localisation of pXG12 and S21 distal to 19.2 is based on more than one X chromosome deletion. In conclusion it is useful to use evidence from one method to confirm order evidence obtained using one of the other methods. Family studies and deletion mapping can be carried out in laboratories without tissue culture facilities and cytogenetic expertise. The potential problems of each method should be considered when interpreting data.

7.1.2 Linkage Analysis of Xq

A genetic linkage map of the X chromosome was published in 1985 (Drayna and White, 1985). The linkage relationships were determined by studying 38 normal families where DNA was available from all four grandparents and which had large sibships, averaging nine children in each. This study included four DNA markers between the centromere and Xq22. A further study (Arveiler et al, 1987) provided a more detailed genetic map of the region Xq11-Xq22. This analysis, using nine DNA markers, was performed on 44 normal families, including 17 families from CEPH (Centre d'Etude du Polymorphisme Humaine, Paris), and 27 families with various diseases.

7.1.3 Physical Mapping

Pulsed field gel electrophoresis was used to map the probes pXG12, p212 and S21 which are linked to XLA. The first aim of the experiments using pulsed field gel electrophoresis was to create a map linking the probes and determine the distances between them. The second aim was to identify potential HTF islands. Information about the distance between the probes and the distance between the probe sequences and HTF islands would affect the strategies used to clone the XLA gene.

Sfi partial digests were used to attempt to link the probes together. A ladder of bands is detected by probes hybridised to partial digests. Two probes are linked when they detect identical bands above a certain band in the ladder. Similarities in smaller band sizes without larger bands in the ladder coinciding, as in the case of the 295kb band detected by pXG12 and S21, simply reflect the fact that many DNA fragments of this approximate size are generated by Sfi digestion and do not demonstrate linkage of the probes. The total length of the genome detected without linking the three probes was approximately 2 megabases.

Calculations assuming an average length of 1kb per HTF island predict that 89% of NotI sites will occur within HTF islands and that 74% of BssHII sites will occur within HTF islands (Lindsay and Bird, 1987). It was also calculated by Lindsay and Bird that there would be 0.12 NotI sites per island and 1.2 BssHII sites per island. Bird has since analysed 19 genes with HTF islands and found 30 NotI sites and 37 BssHII sites indicating that NotI sites occur more often than predicted in HTF islands (personal communication). NotI, BssHII digests and Sfi/NotI, Sfi/BssHII double digests were done to identify potential HTF islands and position them on the physical map.

All three probes hybridised to the limiting mobility in the NotI tracks. Sfi sites must be closer than NotI sites both proximal and distal to the probe sequence as the bands seen after Sfi/NotI digestion were all seen in the Sfi tracks. In order to estimate the distance between a NotI site and the probe partial Sfi digestion could be carried out after NotI digestion. The smallest band to disappear from the Sfi ladder after NotI digestion provides an upper estimate for the distance between the probe and the closer NotI site. However, great care must be taken using this approach to achieve reproducible Sfi partial digests and ensure that absence of a band is not the result of more complete Sfi digestion.

The three probes hybridised to the limiting mobility in the BssHII tracks but, in addition, pXG12 hybridised to a 425kb band and p212 to a 505kb band. A new band of 210kb was detected by pXG12 in the Sfi/BssHII track indicating a maximum distance of 210kb between the probe sequence and a BssHII site. No new bands were detected by

p212 or S21 in the Sfi/BssHII track.

HpaII sites occur approximately every 100 bases in HTF islands but as HpaII has a four base pair recognition sequence only a fifth of HpaII recognition sequences occur in islands. However, HpaII will not digest at many of the sites outside islands because the cytosine in the recognition sequence will be methylated. An Sfi/HpaII double digest was included in the gel shown in figure 5.4. Hybridisation of all three probes to the filter from this gel failed to give a distinct band. All three gave a smear below 120kb. This could either have resulted from degradation of the DNA in this track or reflect that HpaII digestion is not confined to HTF islands.

Long range physical maps have been generated using pulsed field gel electrophoresis around the Duchenne locus on Xp21 (10 megabases) and the cystic fibrosis locus on 7q31 (12 megabases) (Fulton et al, 1989; Burmeister et al, 1988). Four NotI bands were detected using eight probes from Xp21 and five NotI bands were detected using eight probes from 7q31. The smallest NotI band detected was 0.9 megabases and the largest was estimated to be 4.3 megabases. All of these bands are larger than the DNA fragments resolved in the pulsed field gels run in this project. In Burmeister's study the Sfi/NotI double digests gave the same bands as Sfi digests for seven out of the eight probes. However, use of Sfi/NotI digests generated bands of around 300 kilobases for one probe from Xp21 and two of the probes from 7q31. Many endonucleases and double digest combinations were used in the generation of both of these maps and electrophoresis conditions which separated much larger fragments of DNA used.

No recombinations have been observed between two of the probes used in this study but pulsed field gels resolving DNA fragments up to one megabase have failed to link them. It is interesting to note in Fulton's study that the physical distance between D7S97 and D7S73 was 12 megabases, which is equivalent to 12cM, although linkage analysis had given a distance of 3.5cM between these two probes. Conversely recombination events have been noted between the probes D7S97 and D7S101, which were within 350kb of each other on the physical map. Recombinations have been observed between DXS52 and DXS15 but physical mapping of Xq27.3 has placed them within 60kb of each other (Patterson et al, 1987). Thus the correlation between recombination rate and the physical distance between probes is not always good.

The results from pulsed field gel electrophoresis presented here provide the start of a physical map of Xq21.3. Despite the low frequency of recombination in this area the probes have not been physically linked. The most likely distance between pXG12 and S21 from genetic linkage data is 2cM (Arveiler et al, 1987). No recombinations have been observed between pXG12 and p212. In this study pXG12, p212 and S21 detected two megabases of DNA fragments without linking the

probes. This minimum distance map is still consistent with the genetic linkage data. To extend the map, further experiments are needed using conditions which separate larger DNA fragments. It may not be possible to make a physical map of this region using pulsed field gel electrophoresis until more DNA sequences have been isolated which map to Xq21.3.

7.2 Localisation of X-linked Disorders

7.2.1 Choroideraemia

Choroideraemia is an X-linked recessive tapetochoroidal dystrophy causing night blindness by early adulthood, progressive loss of vision and eventually blindness in affected males. Female carriers have normal vision but show progressive degenerative changes in the fundus (Karna, 1986).

Evidence for localisation of the gene which is defective in choroideremia has come from linkage studies and from X chromosome deletions in affected males.

The first linkage study reported linkage to pDP34 with a lod score of 5.78 at $\theta = 0$ (Nussbaum et al, 1985). The next linkage study was carried out in three large families in Northern Finland. This study confirmed the earlier findings with a lod score of 11.44 at $\theta = 0$ for pDP34 and 3.31 for st25 also at $\theta = 0$, but recombinations were observed between 19.2 and the disease locus (Sankila et al, 1987). Recombinations have since been reported between the disease locus and pDP34, st25, and pX65H7 (Lesko et al, 1987). Multipoint linkage analysis suggested that the choroideremia locus is proximal to 19.2 but the data was insufficient to give a localisation proximal or distal to pDP34 (Lesko et al, 1987).

The first male with an X chromosome deletion and choroideremia to be reported also had severe mental retardation, macrocephaly, and a left sided cleft lip and palate. The initial studies showed that this boy had an interstitial deletion of Xq13-q21.3 and that pDP34 did not hybridise to his DNA but S21 did (Schwartz et al, 1986). High resolution banding later showed that the deletion was Xq21.1-21.33 (Schwartz et al, 1988). pX65H7, st25-1, 19.2, pDP34 and p31 (DXYS5) failed to hybridise to his DNA (Schwartz et al, 1988) but S21 and PGK did hybridise to his DNA (Ivens et al, 1988). Patient MB, described in this thesis, was the next reported case. The probes pDP34 and 19.2 failed to hybridise to his DNA (Hodgson et al, 1987), later results have shown that pX65H7 proximally and pXG12 distally did hybridise to his DNA. Two brothers with choroideremia, mental retardation and sensorineural deafness associated with an interstitial deletion of Xq21.2-q21.3 have also been investigated (Schwartz et al, 1988). pDP34 did not hybridise to their DNA but 19.2 did hybridise to their DNA. The XY homologous region probe p31 (DXYS5) gave an altered band size when the DNA was digested by several different enzymes demonstrating that this probe detects the deletion breakpoint in these brothers. A further family was reported in which affected males had choroideremia, mental retardation, obesity, deafness and an interstitial deletion in Xq21 (Ayazi, 1981). pX65H7 and pDP34 were deleted in the males in this family but st25, 19.2 and PGK were all

present (Nussbaum et al, 1987). An additional family with the same combination of choroideremia, deafness and mental retardation has been described in which none of the above loci were deleted and where no deletion was seen on cytogenetic analysis. Phenol enhanced reassociation was carried out using DNA from a 48,XXXX lymphoblastoid line and DNA from one of the males from this family. Sequences characterized from this library, pJL8 and pJL68, were found to be deleted in the males described by Ayazi with the microscopic deletion.

The common probe to all of the microscopic deletions has been pDP34. In addition the gene locus must be proximal to or within the XY homologous region as pX65H7 and pDP34 were deleted in the family described by Ayazi but not st25 and 19.2.

In family HIS the characteristic ophthalmological features of choroideremia were associated with mental retardation and neurological abnormalities but not deafness. Cytogenetic analysis failed to reveal a microscopic deletion. pDP34 hybridised to DNA from the males in this family as did the probe plbD5, which has been deleted in some affected males with submicroscopic deletions, and the probes pJL8 and pJL68, generated from the male with a submicroscopic deletion and choroideremia. In this family the disease did not segregate with the closest polymorphic probe, pDP34, or two more loosely linked probes, cpX289 or 19.2. Recombinations occurred between choroideremia and the linked marker, pDP34, in two out of four offspring. Analysis with LINKMAP of the likelihood of the disease in this family being localised between cpX289 and pDP34 gave a lod of -1.85 using a distance of 8 cM between the two probes. This raises the possibility that this is a previously unreported X-linked condition with a different X chromosome localisation causing neurological and eye problems.

It would be helpful to repeat the ophthalmological examination in this family to confirm the diagnosis and exclude retinitis pigmentosa. In addition audiometry and a detailed neurological assessment should be performed. Unfortunately as the family do not wish to undergo further assessment the situation is unlikely to be clarified until the gene defective in choroideremia has been cloned.

7.2.2 Hypohidrotic Ectodermal Dysplasia

Hypohidrotic ectodermal dysplasia (HED) is an X linked disorder with deficiency of eccrine sweat glands, anodontia or oligodontia with conical teeth, sparse scalp hair and absent body hair in affected males. A distinctive face has been described with a depressed nasal bridge and periorbital pigmentation and wrinkling. Subcutaneous fat is often diminished and over a third of boys have breast abnormalities, including absent or accessory nipples. Affected males may have recurrent chest infections, failure to thrive and life threatening

pyrexias. In one study 50% of affected individuals had a severe illness in early childhood and the mortality was 30% (Clarke, 1987a).

Dental abnormalities were found in 78% of women examined in one series of 46 obligate carriers (Clarke et al, 1987c). Female carriers may also have areas of absent sweat glands, patchy scalp or body hair and breast abnormalities. The starch and iodine whole back sweat test of Happle and Frosch will identify some of the female carriers (Happle and Frosch, 1985).

The first linkage study was carried out by MacDermott, who found linkage between HED and pDP34 with a lod score of 2.7 at $\theta = 0.06$ (MacDermot et al, 1986). Shortly after this Kolvraa investigated families with the probe, pTAK8, which is now known to be on the short arm of the X chromosome, and found a lod of 2.41 at $\theta = 0$ (Kolvraa et al, 1986). Further linkage studies have confirmed a recombination distance of approximately 5 cM between the disease locus and pDP34 (Clarke et al, 1987b; Zonana et al, 1988a; Hanauer et al, 1988). cpX289 has been used in two linkage studies (Zonana et al, 1988a; Hanauer et al, 1988). In the study by Zonana and his colleagues, probes detecting the PGK1 locus were also used and the maximum lod scores were 14.84 for cpX289 at $\theta = 0.01$ and 13.44 for PGK1 at $\theta = 0.02$. One recombination was observed between the disease locus and cpX289 in each of these studies and in the study by Zonana the meiosis also showed a recombination between the disease locus and PGK1. Both of these recombinants are based on the classification of females as carriers. One of these women was classified as a carrier because she gave a history of two missing permanent teeth, her dental records were not available for review and she was edentulous at the time of the study. The other woman had a dry skin and a slight decrease in sweat glands at the age of fifty six. One recombination has been observed between pX65H7 and the disease in the son of an obligate carrier.

A manifesting female with an X;9 translocation was described at the First Human Gene Mapping Meeting in 1972. The AnLy cell line has recently been recognised to be from this female (Zonana et al, 1988b). The breakpoint is at Xq13.1 and DNA analysis has shown the breakpoint to be between cpX289 and PGK1 (Arveiler et al, 1987).

The crucial factors in the interpretation of the haplotype information in family LIT are the diagnosis of HED in the affected male, the assignment of carrier status in his mother and her two sisters, and paternity.

The affected male in this family has many of the classical features of HED but is not completely typical because his whole back sweat test and the finger tip impressions show that he does have sweat pores. Other atypical features are the normal nasal bridge and the normal distribution of subcutaneous fat. His hair, teeth, periorbital pigmentation and absence of nipples, however, all support the

diagnosis. Renal anomalies have not been described before in the condition and were considered as a coincidental finding.

The mother of the affected child had three missing teeth , a pointed right upper lateral incisor and small mandibular incisors. She was noted to have an accessory nipple. Her whole back sweat test was normal.

One sister lacked both upper lateral incisors and the second sister had an absent right upper lateral incisor and an abnormally shaped left upper lateral incisor. Their whole back sweat tests were normal. There was a suggestion of hypodontia on the dental X-rays from these two women and their mother.

In this family there is one boy with major abnormalities and three females with minor abnormalities of teeth and hair. Either the condition in the family is X-linked HED or an autosomal dominant condition with partial penetrance mimicing HED. If the condition is X-linked HED it could be a new mutation in the affected male, a new mutation in his mother, or as thought on clinical grounds all three sisters could be gene carriers.

There is strong support for carrier status in the mother of the affected boy as she has three teeth missing, three abnormal teeth and an accessory nipple. She is heterozygous for the flanking markers, cpX289 and pSPT/PGK. Her paternity was checked using p λ g3. In addition the paternal allele for pSPT/PGK is certain as her mother is homozygous for this probe. The affected male has received her paternal allele for both of these probes. One explanation for this is that she carries a new mutation on the paternal X chromosome. If this is correct then either the two sisters are carriers as a result of gonadal mosaicism, or they are not carriers despite the dental abnormalities. Paternal gonadal mosaicism, however, cannot account for all the data as the two normal males in generation III have inherited the grandpaternal haplotype for this region. This means that if II₁ carries a new mutation on the paternal X chromosome, which is compatible with the mapping information on HED, then her sisters are not carriers.

Alternative explanations for III₁ receiving the grandpaternal allele for cpX289 and pSPT/PGK are a double recombination between these probes or that the disease in this family is at a separate locus. Data from other family members is not consistent with a disease localisation between cpX289 and pSPT/PGK as II₁ has not inherited the same haplotype as her sisters for five informative probes between Xp11-Xq13.

The prior probabilities of the women being carriers of HED are greater than the probability of their dental abnormalities being incidental findings as only 0.6% of the population have three absent permanent teeth and only 2% have two absent permanent teeth, excluding

third molars (Rose, 1966). If all three sisters are carriers of the disorder then a second locus for HED remains the most likely explanation. However, families from Britain, Switzerland, Finland, Denmark, France and the United States have been studied without any suggestion of non-allelic heterogeneity. In addition, the study by Zonana and his colleagues analysed results for the probes cpX289 and pSPT/PGK from thirty six families without finding any evidence for non-allelic heterogeneity.

There are, therefore, two possible explanations of the haplotype results. The first possibility is that the sisters, II₂ and II₃, are not gene carriers and the second possibility is that there is non-allelic heterogeneity. Either of these explanations raises concern about counselling of females in families with HED.

As this family raises the possibility of a second locus for HED the use of cpX289 and pSPT/PGK for clinical testing should be treated with caution in small families where it is not possible to demonstrate that the disease is segregating with the region Xq11-q11.3.

7.2.3 Severe Combined Immunodeficiency

XSCID was mapped to Xq11-13 in a collaborative study between groups in London, Paris and Strasbourg (de Saint Basile et al, 1987). There were no recombinations between cpX289 and the disease in that study. pDP34 and p8 were found to be flanking markers. Two additional probes between these flanking markers have been used in this study to improve the localisation. Three of the families investigated were used in the collaborative study (families KHA, BER, and TEB). Families GWA and HAL have been identified since the preliminary mapping study.

Two recombinations were observed between the disease locus and probes in this study. They occurred between cpX203 and the disease and pDP34 and the disease and so have not provided closer flanking markers. However there is evidence that the disease locus is proximal to pX65H7 from two families with deletions on the X chromosome extending from the XY homologous region to include pX65H7 (Schwartz et al, 1988; Nussbaum et al, 1987). The males carrying the deletions in these families had choroideremia but did not have XSCID (Schwartz et al, 1988; Ayazi, 1981). The order is therefore cpX203 - p8 - (cpX289 - pSPT/PGK - XSCID) - pX65H7 - pDP34.

Lod scores were calculated between XSCID and p8, cpX289, pSPT/PGK and pX65H7 first confining the analysis to obligate carrier females and their male offspring and then repeating the analysis including females whose carrier status was determined by methylation studies. This increased the XSCID - pSPT/PGK lod score from 1.63 to 3.01. As families with XSCID are rare a carrier test for females is useful not only for counselling but also in order to generate more data in linkage studies.

Adding the information from the two new families to the results from the published study increases the two point lod score for XSCID - cpX289 to 7.08. Analysing the results from the five families in this study using LINKMAP increases the lod score at pSPT/PGK to at least 4.

The linkage data for pSPT/PGK and XSCID taken with the mapping information which places pSPT/PGK between cpX289 and pX65H7 provide evidence that it is linked to the disease locus. Its use as an additional polymorphic marker for predictive testing with cpX289 means that 65% of females should now be heterozygous for a linked marker.

7.2.4 X-linked Agammaglobulinaemia

XLA has been mapped to Xq21.3-22 (Kwan et al, 1986; Malcolm et al, 1987). The probes pXG12 and S21 have been used for predictive testing in XLA. In this study linkage has also been demonstrated between p212 and the disease locus with a lod score of 3.74 at $\theta = 0$. The order of the probes in this region is 19.2 - (pXG12 - p212) - S21. This probe can, therefore, be added to those used in predictive testing increasing the number of females who will be informative for a polymorphism detected by a linked marker.

Recombinations have been observed between the disease locus and 19.2 (Kwan et al, 1986; Malcolm et al, 1987; Mensink et al, 1986). This study has not provided a closer proximal flanking marker or a distal flanking marker.

Soon after the initial localisation was reported non-allelic heterogeneity was suggested. This was based on a significant negative lod score in one family for the disease locus at 6cM from 19.2 family (Mensink et al, 1986). This family has since been shown to be a case of gonadal mosaicism with paternal transmission. Excluding this family no recombinations have been reported with either pXG12 or S21.

Family JAM is phenotypically identical to classical XLA and yet recombinations have occurred between the disease and the haplotype of 19.2 - pXG12 - S21 in two out of four meioses in this family. The paternity of III₄ and III₈ were shown to be correct using minisatellite probes and therefore there is no doubt about the haplotype that they inherited from their mother. II₃ and II₄ had three sons who died at less than three years of age. The diagnosis was bronchopneumonia in all three. As male to male transmission never occurs in X-linked disorders, and these three males were almost certainly affected, gonadal mosaicism in II₃ cannot be the explanation for the results. Even if these three males were not affected gonadal mosaicism alone would not explain the results as the affected male has not inherited the complete grandpaternal haplotype.

In this family recombinations have been observed between the disease locus and pXG12 and S21 in one meiosis and 19.2 in a second meiosis out of four meioses. A lod score of -2 is accepted as

excluding linkage. Analysing the probability that the disease locus is between 19.2 and pXG12 or between pXG12 and S21 using LINKMAP the maximum lod scores were -3.8 and -6.5 respectively. The disease locus in this family is, therefore, not between 19.2 and S21. Using the convention of (maximum lod score - 1) to give an upper confidence limit for the estimate of distance between two markers (Conneally et al, 1985) the XLA locus could be 10cM distal to S21 (Malcolm et al, 1987). The results in this family are consistent with non-allelic heterogeneity or a disease localisation distal to S21. If the disease locus in this family is the same as in the other families studied then the XLA locus is distal to S21.

Recombinations with S21 have been reported in the family that has growth hormone deficiency and XLA (Notarangelo et al, 1988). In addition it has been shown that the mechanism of hypogammaglobulinaemia in this family is different from the mechanism in classical XLA. In classical XLA a non-random pattern of X inactivation is found in B lymphocytes but not in T lymphocytes or granulocytes (Conley et al, 1986). In the family with growth hormone deficiency and XLA a non-random pattern of X inactivation was found in T as well as B lymphocytes (personal communication). It would be interesting to do X inactivation studies in purified cell populations in the three obligate carriers in family JAM.

Linkage analysis of this family and the family with XLA and growth hormone deficiency, and the X inactivation studies in classical XLA and the family with XLA and growth hormone deficiency, suggest that there is more than one gene on the X chromosome which, when defective, leads to absence of all immunoglobulin isotypes.

This possibility raises questions about the reliability of using the linked probes for predictive testing. No recombinants had been found prior to this family, with a lod score of 12.9 which is equivalent to 43 informative meioses for S21. A method has been developed which makes allowance for non-allelic heterogeneity in calculations of risk (Lau et al, 1988). The larger the family and the more members tested the smaller the uncertainty attached to predictive testing. Using this method for taking non-allelic heterogeneity into consideration it is reasonable to continue using pXG12 and S21 for predictive testing in XLA.

7.2.5 Pitfalls in the Use of Linked Markers in Genetic Counselling

There are a number of potential pitfalls that can occur in the use of linked markers for genetic counselling. Some of these problems are demonstrated by families HIS, LIT and JAM discussed above.

The information resulting from marker studies will not be correct if there has been an error in diagnosis. It is essential that when families with rare genetic disorders are referred that the diagnosis

is confirmed and that all conditions that can present with similar findings are considered. For example when a family with XLA is referred it is important make certain that the family does not have Hyper IgM as the localisation of the two conditions is different.

It is also important to confirm the carrier status of females in X-linked pedigrees if their DNA results are going to be used in assigning phase. Family LIT demonstrates the difficulties that can arise in trying to decide if females are carriers of X-linked conditions on the basis of clinical findings.

In many clinical situations the information given to families is dependent on correct paternity and this should be made clear to families at the time of counselling.

The possibility of new mutation and germ line mosaicism should also be considered whenever the DNA findings do not fit the expected pattern.

Before considering the final possibility, non-allelic heterogeneity, as the explanation for unexpected results incorrect diagnosis, confusion over sample labelling, non-paternity, new mutation and germ line mosaicism should all be considered.

In view of all of the potential problems that can lead to errors in counselling it would seem wise to restrict the clinical use of linked genetic markers to a limited number of specialised centres.

CHAPTER 8. Discussion: X-inactivation Studies

8.1 Validity of X-inactivation Studies for Determining Carrier Status

Two obligate carriers in this study were shown to have non-random use of the X chromosome in E positive cells.

During the course of this study non-random use of the X chromosome in T lymphocytes of female carriers of X-linked SCID has been demonstrated by another group using somatic cell hybrids (Puck et al, 1987). Purified T cells were fused to HPRT deficient male Chinese hamster lung fibroblasts using polyethylene glycol. The hybrids were grown in HAT medium to select for those retaining the active X chromosome. Hybrid clones that retained two human X chromosomes were grown in medium containing 6-thioguanine, which is toxic to cells expressing HPRT and so selects for clones that have lost the active X chromosome. In this way clones were generated that contained only one human X chromosome which was known to have been active or inactive in the human parent cell. Genomic DNA from the women was tested to find X-linked polymorphisms for which they were heterozygous. Southern blots made using the hybrid DNA were then tested with these probes to see which allele the hybrids retained. Three obligate carriers were investigated and the following results obtained: 22 out of 24 clones with the same X active, 20 out of 21 with the same X active and 17 out of 17 with the same X active. These results were significantly different from the normal random X inactivation pattern. In addition in one of these three carriers they were able to show that the haplotype of the inactive X was tracking with the mutation in the family. This method provided strong evidence for a non-random X inactivation pattern in mature T cells of carriers of the X-linked disease. However the procedure takes months for each subject investigated, is expensive and labour intensive and therefore it is not practical as a routine test.

As the non random pattern of X inactivation in T cells of female carriers has now been demonstrated by two independent methods it seems reasonable to give genetic counselling on the basis of X inactivation patterns. DNA methylation analysis is the more practical of the two methods for carrier testing for XSCID. The basis of the method is that when an X-linked gene is required for cell survival, the normal gene will always be found on the active X chromosome and the defective gene on the inactive X chromosome in the mature cell population, even though initial X inactivation is random.

8.2 Potential Errors Associated with the Use of X Inactivation

An incorrect diagnosis would be reached if a carrier female had a random pattern or a normal female had a non random pattern as assessed by methylation analysis. If female carriers of the X-linked disorder

always have a non random distribution of active X chromosomes in the mature T cell population then the only cause of a carrier female having an apparently random distribution would be if digestion with the methylation sensitive enzyme was incomplete. In order to avoid this happening an excess of the methylation sensitive enzyme was added and in addition the experiment was performed twice for each woman before any result was given to the family.

As X chromosome inactivation is a random event mature cell populations from a number of females would have a mean of 50% of cells having paternal X inactive and 50% having the maternal X inactive. However the results from a group of women are scattered around the mean in a normal distribution. The possibility of erroneously saying that someone has a non random pattern and is therefore a carrier depends on the distribution around the mean and the sensitivity of the test.

To assess the distribution around the mean neutral polymorphisms have been studied to see how many heterozygous females express only one allele in mature cell populations. Fialkow studied 41 subjects who were heterozygous for the G-6-PD isoenzymes A and B (Fialkow, 1973). Purified lymphocytes were investigated in 20 of these women. The mean percentage of enzyme A in lymphocytes was 48, $SD \pm 11.39$, range 25%-75%. The range for two standard deviations is 25-71% and for three standard deviations is 19 - 77%. The sensitivity of the isoenzyme analysis is not clear but both alleles were identified in the lymphocyte fraction of all the women studied.

Fearon and Vogelstein have published methylation analysis results for E positive cells from 2 women using pSPT/PGK and from one woman using HPRT (Fearon et al, 1987). Lymphocytes from the E positive cell fraction have been investigated with pSPT/PGK from two women and with HPRT from one woman in this study. In these six women random use of the X chromosome was observed in the T lymphocytes. Vogelstein has studied DNA from normal tissues, including lymphocytes, from 40 women using the pSPT/PGK and 42 women using the HPRT probes (Vogelstein et al, 1987). In two of the women heterozygous for the polymorphism detected by pSPT/PGK the density of one of the bands decreased by 80% and the other by only 20% as assessed by dosimetry. In one of the 42 women heterozygous for the polymorphism detected by HPRT the intensity of one band decreased by 85% and the intensity of the other band decreased by 15% in several tissues examined. However in all 82 women two bands were present after the addition of the methylation sensitive endonuclease.

8.3 PGK Linkage in Combination with X Inactivation

It has been shown in this thesis that pSPT/PGK is linked to the XSCID disease locus in families which have a clearly X-linked pedigree. It has also been shown that analysis of X inactivation patterns with pSPT/PGK can be used for carrier testing in heterozygous females.

All of the CpGs in the PstI/BstXI fragment of DNA detected by pSPT/PGK are unmethylated on the active X chromosome but methylated on the inactive X chromosome. Thus when HpaII is added the DNA from the inactive X chromosomes is not digested but the DNA from the active X chromosomes is digested into much smaller fragments. The allele which is lost after the addition of HpaII in a heterozygous female with a non-random pattern of X inactivation, therefore, represents the active X. The allele that remains after the addition of HpaII represents the inactive X i.e. the X chromosome carrying the mutation. As pSPT/PGK is linked to the disease it is possible to assign phase in a female shown to be a carrier by methylation analysis using pSPT/PGK without analysing DNA from any other member of the family. pSPT/PGK can then be used as a linked probe for prenatal diagnosis of the condition. A male fetus inheriting the band that remained after the addition of HpaII during methylation analysis is at high risk of being affected and a male inheriting the allele which was lost after the addition of HpaII is at low risk of being affected.

This unique situation of using the same probe in a carrier detection test and as a linked probe for predictive testing is especially useful when the consultand may carry a new mutation. In this situation one cannot assign phase using DNA from the consultand's father. In addition in these families with sporadic cases the affected males often die shortly after presentation. In the three families with sporadic cases presented here the affected males all died before tissue was stored for extraction of DNA and there were no normal male offspring who could be used for phase assignment. In pedigree HAL both affected males had died without tissue being stored for DNA extraction and none of the three carrier sisters have any normal male offspring who could be used to assign phase and their father is deceased.

Prenatal tests could not be offered in pedigree LAW or pedigree HAL without the use of pSPT/PGK for both carrier detection and for assignment of phase.

8.4 Affect on Counselling

Developing a carrier test for XSCID has helped in the genetic counselling of women at risk in X-linked families where the key females were not heterozygous for linked probes. However it has been most useful when the pattern of inheritance was not clear from the pedigree. This is demonstrated by pedigrees HAL and MOR which have a

similar structure with a proband having two affected male children in each family.

In pedigree HAL the proband was shown to be a carrier of the X-linked form of the disease. This not only allowed better counselling of the couple but has also led to more accurate counselling of the extended family. The two sisters had previously been told that they might be at risk of having affected children but counselling was difficult because the disease could have been autosomal or X-linked and, if X-linked, the proband could represent a new mutation. Following methylation analysis both sisters have been given precise information and offered prenatal testing.

In pedigree MOR the proband had a random X inactivation pattern in her T cells suggesting that the disease was autosomal recessive in this family. Again this allowed more accurate genetic counselling to the extended family. In this situation the proband's sister was reassured that her own risk, and her daughter's risk, of having affected children is extremely small as although they may be carriers of the autosomal recessive form the chance of an unrelated individual being a carrier is less than one percent.

The proband in pedigree EIS who has had a single affected male had a non-random pattern of X inactivation and is therefore a carrier of the X-linked form of the disease. However, the chromosome carrying the mutation is the X that she has inherited from her father. She therefore carries a new mutation and her own mother is not a carrier of the disease. The proband's sister can only be carrier of the disease if there is significant germ line mosaicism in their father. Whilst germ line mosaicism has been demonstrated in a Duchenne family (Darras and Francke, 1987) and also in the XLA family discussed earlier it is still a rare event. The risk of the proband's sister being a carrier of the disease is therefore low and she has been reassured.

When the mother of a sporadic case does not have a non-random pattern of X inactivation the counselling becomes more difficult because there are two possible explanations for the finding. The affected child could have had autosomal recessive SCID or could have had X-linked SCID as a result of a new mutation. Considering the 4:1 ratio of males to females affected the ratio of X-linked to autosomal recessive must be 3:1 and hence the probability of a single male having the X-linked type is 75%. As with all X-linked lethal conditions the proportion of sporadic males carrying new mutations is theoretically one third. Therefore in a family with one affected male and no normal sons the ratio of autosomal: X-linked inherited: X-linked new mutation is 1:2:1. When the possibility of the child having inherited the X-linked form has been excluded, as in pedigree SPE, the possibilities of autosomal recessive and X-linked new mutation are

equally likely. One can therefore give a recurrence risk of 1 in 8 of a child being affected. If, as in this family, the child did not have adenosine deaminase deficiency the balance is more in favour of a new mutation than autosomal recessive inheritance. However the couples are still at significant risk of having another affected child but early prenatal diagnosis cannot be offered. Even in this situation the information is useful when counselling female relatives of the proband. The probability of a female relative having an affected child is low, whether the affected boy carries a new mutation or has the autosomal form, and reassurance can be given.

8.5 Usefulness of Test

Methylation analysis using pSPT/PGK and HPRT allows carrier detection in women heterozygous for the polymorphisms detected by these probes. Vogelstein estimated that over 50% of American females would be heterozygous for one or other probe (Vogelstein et al, 1987). The information content for pSPT/PGK in this study was the same as that observed by Vogelstein. However using the HPRT allele frequencies observed in this study only 11% of females would be heterozygous compared with a figure of 29% given by Vogelstein. Using the figures obtained in this study 40% of women would be heterozygous for one of the probes and therefore 60% of women referred because they have had an affected male cannot be offered the test.

Recently it has been suggested that the probe M27 β (DXS255) may also detect methylation differences between the active and inactive X chromosome (Y Boyd, personal communication). The advantage of this probe over the probes developed by Vogelstein is that it detects a length polymorphism resulting from tandem repeats which vary considerably in number between individuals. It has been estimated that over 95% of women are heterozygous for the length polymorphism detected by this probe. If these methylation differences are confirmed then carrier detection could be offered to most women using methylation analysis with this probe.

8.6 Expression of the Gene Defective in XSCID

The E positive fraction of cells in all obligate carriers and women known to be at high risk because of their family history and linked probe analysis had a non-random pattern of X chromosome usage on methylation analysis. This cell fraction contained on average 84% (range 74 - 93%) T lymphocytes as determined by indirect immunofluorescence following staining with CD3 monoclonal antibody. HNK1 positive natural killer cells accounted for the remaining cells with B lymphocytes and monocytes accounting for less than 1% of the cells.

When the purity of a cell population is 75% then two bands could

be observed after the addition of the methylation sensitive endonuclease when the predominating cell type is using only one X chromosome if the contaminating cells use the X chromosome randomly. However, a non-random pattern would never be seen as an artefact of cell mixing. Therefore, although the T lymphocyte enriched population contained some contamination the conclusion that there is non-random use of the X chromosome in T lymphocytes is valid. The fact that all of the E positive fractions gave only one clear hybridising band after the addition of HpaII suggests that there is also selection for HNK1 positive natural killer cells with the normal gene on the active X chromosome.

The only cell population in which a random pattern of X chromosome usage was found was the granulocyte fraction. As the granulocyte fractions were 99% pure these results could not have been an artefact of cell mixing. A random pattern was observed in the granulocyte DNA from four women but a non-random pattern was observed in the DNA from two of the women.

The findings in E negative cells and in the monocyte enriched population are strongly suggestive that the gene is expressed in monocytes.

It was not possible to isolate sufficient B lymphocytes for DNA extraction from the venous blood sample taken and so multiple EBV transformed lines were established from the subjects. The results, therefore, apply to B lymphocytes carrying the EBV receptor. EBV transformed lines from normal individuals can become monoclonal after continuous culturing. In a recent study Migeon et al (Migeon et al, 1988) showed that 50% of EBV lines from women heterozygous for electrophoretic variants of G6PD expressed only one of the G6PD alleles by the tenth subculture, within eleven weeks of establishing the culture. In Migeon's study no lines assayed prior to subculture expressed only one G6PD allele. In this study DNA was isolated when there were 5×10^6 cells in each line to minimise the possibility of lines becoming oligoclonal or monoclonal. An alternative method of ensuring that the lines were not clonal when the X-inactivation studies were carried out would have been the use of immunoglobulin gene probes to investigate immunoglobulin gene rearrangements (Arnold et al, 1983). The presence of clear bands in addition to the germline band would indicate clonal expansion of EBV transformed cells. The conclusion, however, is based not on finding use of only one X chromosome in individual EBV lines but on the finding that for each subject the same X chromosome was active in each culture with a non-random pattern, and that this was the same X as in the corresponding T lymphocytes. In subject 2, ten lines were tested and all ten retained only one active X chromosome, the same X as in the corresponding T lymphocytes. The loss of the mutant X in all ten lines is

statistically significant and cannot be explained by the lines developing oligoclonality. The gene is therefore expressed in B lymphocytes with the EBV receptor in this subject. Results were also obtained for nineteen EBV transformed cell lines from subjects 3, 5 and 6. Non-random X chromosome usage was observed in eight of these cell lines and again the active X chromosome was the same in all these lines as in the corresponding E positive cells indicating that the gene is expressed in the B lymphocytes of these subjects. However, two bands were seen after the addition of the methylation sensitive enzyme in eleven of the nineteen EBV transformed lines showing that some circulating B lymphocytes do have the defective gene on the active X chromosome.

There was variation between the subjects for the X-inactivation patterns observed in granulocytes, EBV lines and DNA extracted from venous blood. Comparing the two obligate carriers a non-random X inactivation pattern was found in DNA extracted from venous blood in subject 7, but a random pattern was observed in DNA from venous blood and granulocytes from subject 1. Both of these women came from large families in which the disease segregated with polymorphic markers assigned to Xq11-13. As the genetic evidence argues against there being more than one locus for XSCID it seems that the gene defective in this disorder is expressed in B lymphocytes and granulocytes, but that this has a variable affect on cell survival. This is supported by the differences in the granulocyte X inactivation patterns observed in subjects 4, 5 and 6 who were members of the same family.

Conley has investigated expression of the gene in different cell types using the somatic cell hybrid method (Conley et al, 1988). She found a non-random pattern of X chromosome usage in T lymphocytes and B lymphocytes but a random pattern in monocytes. She did not investigate granulocytes. The findings presented here showing that some B lymphocytes do have the defective gene on the active X chromosome differ from those of Conley. This difference may be explained by the fact that Conley cultured EBV transformed lines until there were 50×10^6 cells before using them to make somatic cell hybrids. It is therefore possible that at the time that the hybrids were made selection had occurred and that cultured cells no longer reflected the phenotype of the circulating B lymphocytes in the subjects. Conley's finding of random X chromosome usage in monocytes also differs from the results presented here. Neither result is based on analysis of pure monocyte populations. As discussed above a random pattern of X inactivation can be an artefact of contamination but a non-random pattern cannot be caused by cell mixing.

The methylation method of determining X inactivation which we have used is not able to distinguish between complete absence of cells expressing the mutant gene and a few surviving cells. The somatic cell

hybrid method used by Conley and her colleagues is also inefficient at detecting a low proportion of cells carrying the mutant gene on the active X chromosome. Similarly, neither method distinguishes between completely random X chromosome usage and a system in which survival is biased towards cells with one of the X chromosomes active. In the case of the methylation method this is because dosimetry of bands is unreliable and in the somatic cell hybrid method because of the statistical nature of the sampling.

A mutant gene leading to an immunodeficiency is unlikely to affect the initial pattern of X inactivation. A non-random X inactivation pattern in a mature cell population could be a result either of a failure of cells without the gene to become fully differentiated due to the lack of a regulatory function or differential survival of the cells. The gene defective in XLA is likely to be in the first category as pre-B cells are found in affected males but no circulating B cells and as the only haematopoietic cells to show non-random use of the X chromosome in carrier females are the B lymphocytes. Differential survival of cells with the normal gene on the active X is a more likely explanation for the findings in XSCID as non-random use of the X chromosome has been observed in cell types which are present in the affected males.

Differential survival has been found in Lesch-Nyhan disease which is caused by deficiency of hypoxanthine phosphoribosyl transferase, an enzyme in the purine salvage pathway. Female carriers of Lesch Nyhan disease have a non-random pattern of X inactivation in erythrocytes and lymphocytes, demonstrating that differential survival can result from a defective gene in a metabolic pathway, but they have the normal random pattern in fibroblasts (Nyhan et al, 1970). It would be interesting to find out if female carriers of XSCID had random use of the X chromosome in non-haematopoietic cells but even if random use was found in fibroblasts this would not exclude the possibility that the disease resulted from a defect in a gene in a metabolic pathway.

When an X-linked immunodeficiency results from a mutation in a gene involved in a process specific to the immunological function of the mature cell and not necessary for its differentiation or survival one would expect to find random use of the X chromosome in the mature cells of a female carrier. This is the case in female carriers of chronic granulomatous disease. The mutation in this disease is in the cytochrome b_{-245} gene. Cytochrome b_{-245} is not required for cell growth or survival but is essential for bacterial killing activity. When cytochrome b_{-245} is absent the oxidase activity that normally accompanies phagocytosis is absent and hence granulocytes from patients fail to reduce nitroblue tetrazolium. In female carriers two populations of cells are seen, some granulocytes are normal but others fail to reduce nitroblue tetrazolium and this is used as a carrier

test. As non-random use of the X chromosome has been found in females carrying XSCID the abnormality is unlikely to be in a pathway required specifically for the immune function of the cells.

The pattern of expression found in XSCID is consistent with the gene causing XSCID being a metabolic enzyme in a pathway to which T lymphocytes are particularly sensitive, in a similar way to adenosine deaminase in the autosomal form.

Summary

XLA and XSCID are disorders of the immune system which are inherited as X-linked recessive conditions. Females carrying these two conditions have no known immunological or biochemical abnormalities. XLA has been localised to Xq21.3-q22 (Malcolm et al, 1987) and XSCID has been localised to Xq11-q13 (de Saint Basile et al, 1987). The DNA probes linked to the disorders have been used for carrier detection and prenatal diagnosis.

A genetic map of the region is essential for localising the disorders more precisely and for planning strategies to clone the genes. The first aim of this thesis, therefore, was to clarify the order of probes assigned to proximal Xq and make a physical map of the probes linked to XLA and XSCID. Fourteen DNA markers detecting polymorphisms were studied in four families selected because of recombinations on Xq and in a male with an Xq deletion. The investigation of both recombinant chromosomes and the X chromosome deletion provided information about the order of probes on proximal Xq. Studies of somatic cell hybrids have clarified the order of these probes further. Combining all available information the order of the probes is (pTAK8 - M27 β) - cpX203 - p8 - cpX289 - pSPT/PGK - pX65H7 - pDP34 - 19.2 - (pXG12 - p212) - S21. The localisation of cpX203 proximal to p8 has not yet been confirmed by other studies. The probes G3-1 and pA13.R1 have been shown, in this study, to detect the same polymorphism but their position in the map is uncertain. The order of pXG12 and p212 and the order of pTAK8 and M27 β is not known.

Pulsed field gel electrophoresis was used to generate a physical map around the probes pXG12, p212 and S21. The most likely distance between pXG12 and S21 from genetic linkage data is 2cM, which is equivalent to two megabases. The total length of the genome detected in this study without linking these three probes was two megabases. Further experiments are required to make a physical map linking these probes and clarify whether there is a low recombination rate in this region of the X chromosome.

X-Linked Agammaglobulinemia

XLA has been mapped to Xq21.3-q22 and the probes pXG12 and S21 have been used for predictive testing. Recombinations have been observed between the disease locus and 19.2 but no close distal flanking marker has been identified. From previously published data the XLA locus is distal to 19.2 but could be proximal to pXG12, between pXG12 and S21 or up to 10cM distal to S21. S21 was the most distal of the fourteen DNA markers ordered in the first part of the study and, therefore, in this group of probes there were no probes that could be tested to find distal flanking markers. In the mapping section of this thesis p212

was shown to be between 19.2 and S21. Linkage analysis between p212 and XLA gave a two point lod score of 3.74 at $\theta = 0$ and p212 is, therefore, an additional linked probe that can be used for predictive testing in this disorder.

Recombinations were observed between the disease locus and pXG12 and S21 in one family, pedigree JAM. Analysis of the data with the LINKMAP section of LINKAGE 4.7 excludes linkage of the disease between 19.2 and S21 in this family, but does not exclude linkage 3.3cM distal to S21. If the disease locus in this family is the same as in the other families studied then the XLA locus is distal to S21. An alternative explanation of the results in this family, however, is non-allelic heterogeneity. This would be resolved by finding XLA families where the disease locus segregates with pXG12 but not S21, indicating that the disease locus is proximal to S21 and thus supporting non-allelic heterogeneity, or finding more distal markers and testing whether they segregate with the disease in this family.

X-Linked Severe Combined Immunodeficiency

XSCID has been mapped to Xq11-q13 in a collaborative study between groups in London, Paris and Strasbourg. Recombinations were observed between the disease locus and p8 proximally and pDP34 distally. In the additional families investigated in this study recombinations were observed between the disease and cpX203 and the disease and pDP34. These recombinations did not provide closer flanking markers. However, the XSCID locus must be proximal to pX65H7 as males with deletions extending from the XY homologous region and including pX65H7 have been described in two families. The affected males in these families had choroideremia and mental retardation, but did not have XSCID.

In the probe ordering section of this study pSPT/PGK was shown to be between cpX289 and pX65H7. Linkage analysis between pSPT/PGK and XSCID gave a two point lod score of 3.01 at $\theta = 0$ when including females whose carrier status was determined by X Inactivation patterns in the analysis. This probe can be used as an additional linked marker for XSCID, making predictive testing possible in more families.

There are X-linked and autosomal recessive forms of severe combined immunodeficiency. This caused difficulties in counselling couples who had one affected son and where there was no previous family history of the disorder. It has been shown in this thesis that female carriers of XSCID have a non-random X inactivation pattern in E positive cells. This has also been demonstrated by Conley using somatic cell hybrids. We have used methylation analysis of X inactivation patterns for carrier detection in women in X-linked pedigrees not informative for the linked probes and in families where the inheritance pattern was not clear. Forty percent of women at high risk of being carriers of XSCID were heterozygous for the polymorphism

detected by PGK or the polymorphism detected by HPRT and could be offered the test.

In addition as the probe pSPT/PGK, which was used for analysis of X inactivation patterns, was linked to the disease locus carrier detection and assignment of phase could be carried out in one procedure. This allowed assignment of phase in women with no living male offspring and who could be carrying a new mutation.

It has been thought that XSCID results from a defect in a T lymphocyte specific gene because the phenotype is predominantly a lack of T lymphocytes and because host B lymphocytes produce functional antibody following transplantation and engraftment of T lymphocytes. Finding a non-random pattern of X chromosome usage in a mature cell population implies that the defective gene is expressed in that cell type and this technique was used to investigate gene expression. Non-random X chromosome usage was found in T lymphocytes, B lymphocytes, monocytes and granulocytes. These findings do not support the hypothesis that XSCID results from a defect in a T lymphocyte specific gene or a gene specific to lymphocyte development. The pattern of expression suggests that the underlying defect in XSCID is in a general metabolic pathway rather than a pathway specific to lymphocytes.

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Appendix 1

Locus	Probe	Enzyme	Polymorphism	Allele Frequency	pic	Wash Stringency
DXYS1	pDP34	TaqI	11/12 15 Y specific band	0.6/0.4	0.48	0.5 X SSC
DXS159	cpX289	PstI	5.5/1.6	0.67/0.33	0.44	0.5 X SSC
DXS255	M27B	multi-allelic			>0.90	0.5 X SSC
DXS94	pXG12	PstI	6.5/7.2	0.60/0.40	0.48	0.5 X SSC
DXS17	S21	TaqI	2.0/2.2 constant bands 4/1	0.35/0.65	0.46	0.5 X SSC
DXS72	pX65H7	HindIII	0.7/7.2	0.55/0.45	0.495	0.75 X SSC
DXS88	G3.1	BglII	4.6/3.5	0.78/0.22	0.34	0.5 X SSC
DXS87	pA13.R1	BglII	4.6/1.1	0.61/0.39	0.47	0.5 X SSC
DXS1	p8	TaqI	15/9	0.84/0.16	0.27	0.1 X SSC
DXS3	19.2	TaqI	2.95+2.15/5.1	0.62/0.38	0.47	0.5 X SSC
DXS146	pTAK8	XbaI	3.3/5	0.64/0.36	0.46	0.5 X SSC
PGK1	pSPT/PGK	BglI	12/9	0.75/0.25	0.38	0.2 X SSC
DXS106	cpX203	BglII	1/5.8	0.64/0.36	0.46	0.75 X SSC
DXS178	p212	TaqI	3.2/1.8 constant band 7	0.70/0.30	0.42	0.5 X SSC
HPRT	HPRT-800 HPRT-600 pλg3	BamHI multi-allelic	24/12 multi-allelic	0.94/0.06	0.11 0.98	0.1 X SSC 0.2 X SSC

This table gives the endonucleases, polymorphic band sizes and the information content for the probes used in this study. The information is taken from the Ninth Human Gene Mapping Meeting. There is no data for HPRT-600 and HPRT-800 in HGM 9 and the allele frequencies quoted are from this study. The information content of M27B and pλg3 are taken from the original references (Fraser et al, 1987; Jeffreys et al, 1988). The band sizes for G3-1 and pA13R1 are those found in this thesis and not those given in HGM 9 which are incorrect. It was agreed at the HGM9 Meeting that cpX289 should be reassigned to DXS159 although in the documentation the locus designation has not yet been changed from DXS162. The final wash stringencies which were found to give the clearest results are given in the final column, all of these were done at 65°C. The HPRT probes and p212 were preannealed with human DNA and pSPT/PGK preannealed with salmon sperm DNA as described in section 4.5.5.

Appendix 2

Solutions

TE

10mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

SSC (20X)

3M sodium chloride
0.3M tri sodium citrate

pH adjusted to 7.0 using HCl

Denhardt's solution (100X)

2% bovine serum albumin (pentax fraction V)
2% ficoll
2% polyvinyl pyrrolidone

Hybridisation Solution

10X Denhardt's solution
4 X SSC
10µg/ml poly(A)
50µg/ml denatured salmon sperm
1% SDS

LBroth

1% tryptone
0.5% yeast extract
0.5% NaCl
0.1% glucose
pH adjusted to 7.4 using 1N NaOH

LB Agar

1% tryptone
0.5% yeast extract
0.5% NaCl
0.1% glucose
1.5% agar
pH adjusted to 7.4 using 1N NaOH

Gey's solution

Solution A:

NH ₄ Cl	35.0g
KCl	1.85g
Na ₂ HPO ₄ .12H ₂ O	1.5g
KH ₂ PO ₄	0.119g
Glucose	5.0g
Phenol Red	0.005g
Gelatine	25.0g
1 litre double distilled water	

Solution B:

MgCl ₂ .6H ₂ O	4.2g
MgSO ₄ .7H ₂ O	1.4g
CaCl ₂	3.4g
1 litre double distilled water	

Solution C:

NaHCO ₃	22.5g
1 litre double distilled water	

All three solutions are stored in 20ml aliquots at 4°C. Gey's solution is freshly prepared by mixing 7mls of sterile water with 2mls of solution A (warmed at 37°C prior to use to melt the gelatine) 0.5 mls of solution B and 0.5mls of solution C.

Culture medium

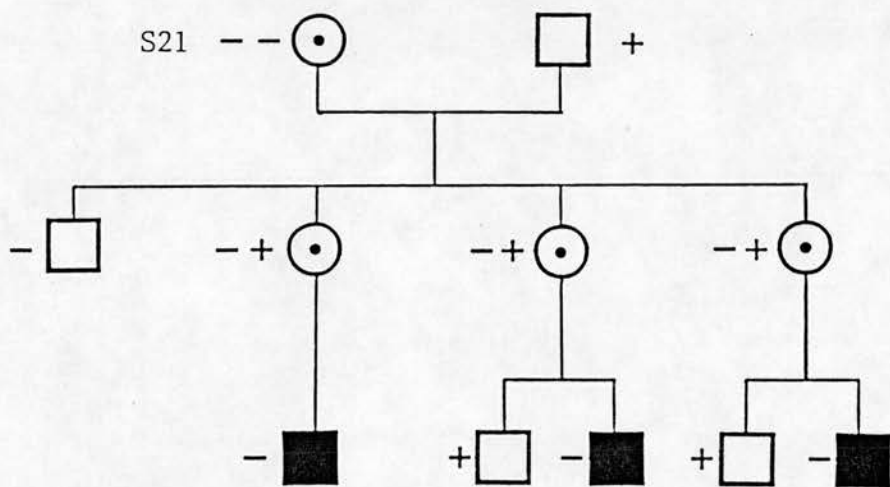
RPMI 1640 supplemented with 25mM Hepes (Gibco)
2mM glutamine
10% FCS
50µg/ml gentamicin

Wash medium

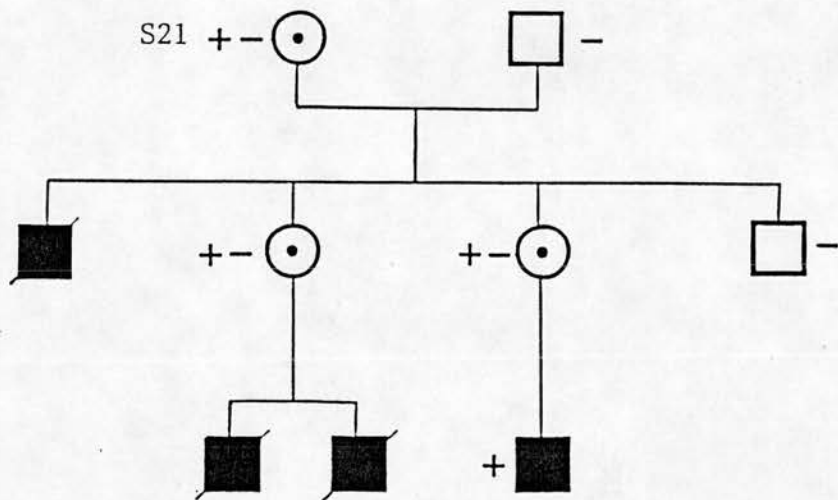
RPMI 1640 supplemented with 25mM hepes (Gibco)
5% FCS unless otherwise stated

Appendix 3
X-linked Agammaglobulinaemia Pedigrees

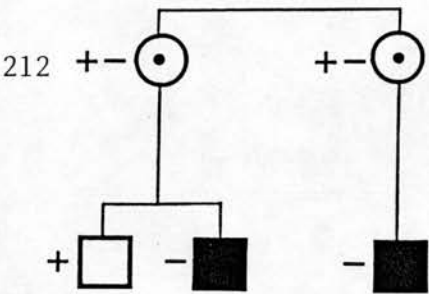
Family CAS



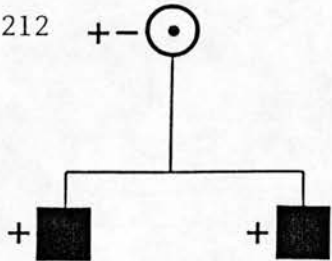
Family MON



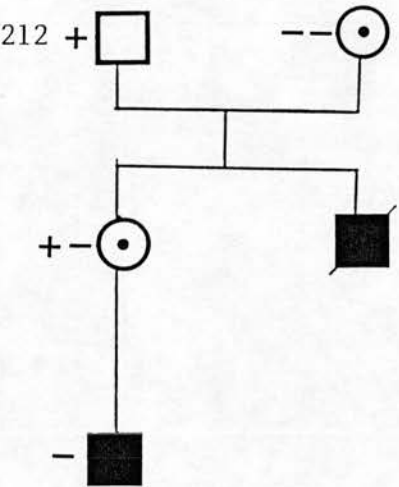
Family LUC



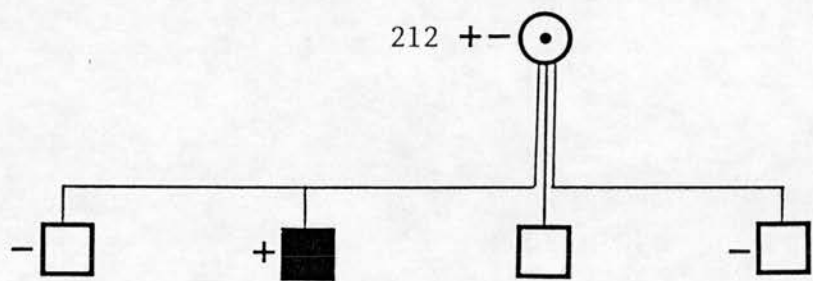
Family COX



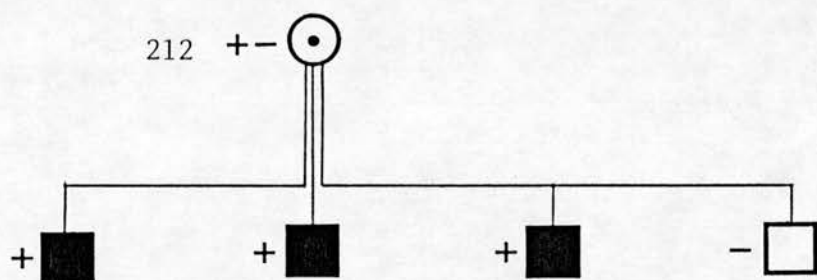
Family FOX



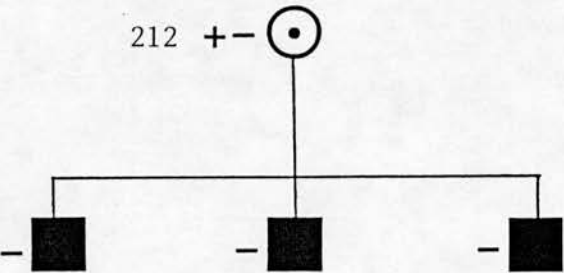
Family RMS



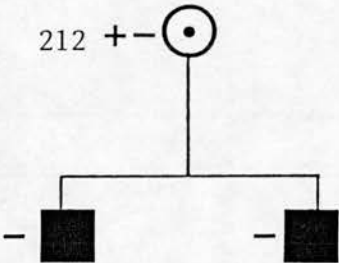
Family GER



Family MAN



Family WIL



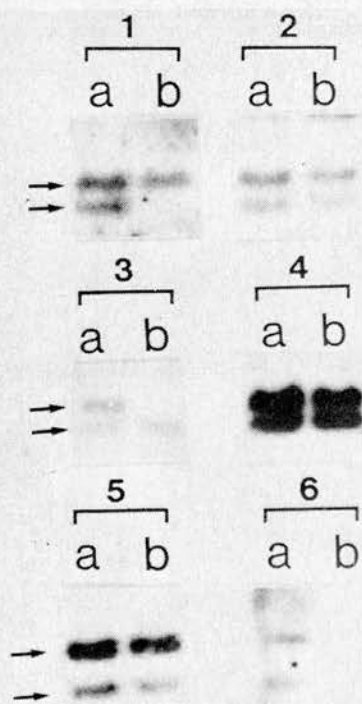


Fig 7—Pedigree family 6.

In pedigree 4 the proband who had had two affected sons had a non-random pattern of X inactivation in her T cells. Methylation analysis showed that her sister, II₄, is heterozygous and after the addition of *Hpa*II the 1.05 kb band was lost (fig 7, lanes 3a and 3b). The disease in this family must therefore be X-linked and both these sisters carriers. The third sister, II₆, is also heterozygous but both bands remained after the addition of *Hpa*II and she is therefore not a carrier (fig 7, lanes 4a and 4b).

The subject in pedigree 5 has had two affected sons and one normal son; there is no previous family history and the disease could therefore be either X-linked or autosomal. She is heterozygous, and after the addition of *Hpa*II both bands remain (fig 7, lanes 5a and 5b), which suggests that she is a carrier of one of the autosomal recessive forms of SCID and therefore her sister and niece will not be at risk of having affected offspring.

Sporadic Cases

For II₂, in pedigree 6, only the 1.05 kb band remained after the addition of *Hpa*II. She therefore has a non-random population of T lymphocytes and is likely to be a carrier of the X-linked form of SCID.

The results of all the X-inactivation studies on known and possible carriers of X-linked SCID are summarised in the table.

Discussion

We have established that DNA methylation analysis can be used for carrier detection of the X-linked form of SCID. The basis of the method is that when a gene is required for cell survival, the normal gene will always be found on the active X chromosome and the defective gene on the inactive X chromosome in the mature cell population, even though initial X inactivation is random. In X-linked SCID the mature T lymphocytes therefore show a non-random X inactivation pattern. This finding is of clinical importance

because carriers of SCID do not have any known biochemical or immunological abnormalities except for carriers of adenosine deaminase deficiency, in which case measurement of the enzyme may be useful.

The non-random pattern of X inactivation has been demonstrated in T lymphocytes in X-linked SCID previously by the use of somatic cell hybrids,⁷ but this method would clearly not be practical as a routine clinical test. Similarly, a protein polymorphism of glucose-6-phosphate dehydrogenase has been used to investigate X-inactivation patterns in X-linked disorders,⁸ but few caucasian females are informative for this polymorphism. Over 50% of caucasian females are informative for the HPRT and PGK polymorphisms³ and this method will therefore be clinically useful.

In families with clear X-linked pedigrees the use of linked probes is considerably easier. However, in a number of families this approach is not possible because there are no surviving males to give linkage phase or because key females are not heterozygous for the probe. In these situations methylation analysis will be of great value.

In addition, in families in which a couple have had several affected males, methylation analysis distinguishes the X-linked form of SCID from recessive varieties. The method can also be used in families referred for investigation after a diagnosis of SCID has been made in a single male infant. Where there is no prior family history it may be possible to show that the mother is a new mutation by demonstrating that it is the paternal X that is inactive in all of her T lymphocytes.

All of this information is valuable in giving accurate genetic counselling to an extended family. The options available to those at risk who ask for prenatal diagnosis include first trimester chorion villus sampling for fetal sexing, prediction in males using the linked probes in some families, or fetal blood sampling at 18 weeks' gestation for absolute diagnosis.⁹ Apart from the immediate clinical application of the test, the finding of more X-linked families will be useful in refining the linkage data in this disorder.

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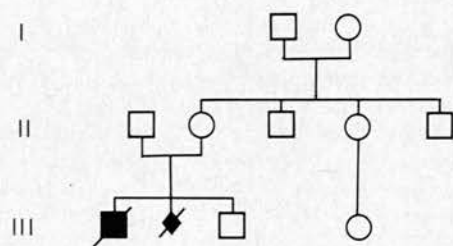


Fig 5—Pedigree family 5.

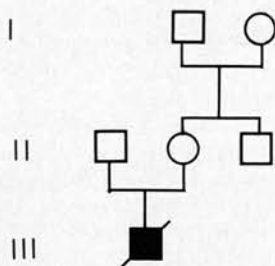


Fig 6—Analysis of X chromosome inactivation patterns.

T lymphocyte DNA has been digested with *PstI/BstXI* in track a and digested with *PstI/BstXI/HpaII* in track b of each sample. Sample 1 is from an obligate carrier, pedigree 1, and sample 2 is from her daughter. Sample 3 is from subject II₄, pedigree 4, and sample 3 her sister, subject II₆. Sample 5 is from subject II₂, pedigree 5. Sample 6 is from subject II₂, pedigree 6.

been investigated. In pedigree 5 (fig 5) a boy diagnosed as having SCID died; the second pregnancy was terminated because fetal blood sampling revealed an affected male fetus; and prenatal diagnosis in the third pregnancy revealed a normal male. In pedigree 6 (fig 6) there was an affected boy with no previous family history.

Cell Separation

Mononuclear cells were isolated from 50 ml of heparinised blood on a Ficoll-Hypaque density gradient. The mononuclear cells were then separated into T-enriched and T-depleted fractions by rosetting with aminoethylisothiuroniumbromide-hydrobromide (AET)-treated sheep red cells (E). The purity of the E-positive fraction of cells was assessed with the monoclonal antibodies UCHT1(CD3), HNK1, UCHM1(CD14), and B1(CD20). 75–85% of the E-positive cells stained with UCHT1 and 7–23% stained with HNK1. B1 and UCHM1 were less than 1% with the exception of one cell separation (pedigree 5, subject II₂ where 4% of the AET-positive fraction were stained by B1).

DNA Analysis

DNA was extracted from the E-positive fractions by standard methods. 15 µg of DNA was digested with *PstI* (Northumbria Biologicals Ltd) in 50 mmol/l "tris"-HCl pH8, 10 mmol/l MgCl₂, 50 mmol/l NaCl at 37°C for 3 h in a reaction volume of 80 µl. *BstXI* (Northumbria Biologicals Ltd) was added, and then the sample was incubated at 56°C for 3 h. The nucleic acids were then precipitated by adding 40 µl 7.5 mmol/l ammonium acetate and 240 µl ethanol with 20 µg glycogen (Boehringer) as a carrier. After 1 h at -20°C the sample was centrifuged in a microfuge for 30 min. The DNA pellet was dried in vacuo and then resuspended in 60 µl of 10 mmol/l "tris"-HCl pH8, 1 mmol/l edetic acid, pH8. This was divided into two equal portions. One portion was digested with *HpaII* (New England Biolabs Inc) for 3 h at 37°C in 6 mmol/l KCl, 10 mmol/l "tris" 7.4, 10 mmol/l MgCl₂, and 10 mmol/l mercaptoethanol. The samples were run on 0.8% agarose gels and transferred onto nylon membranes ('Hybond N', Amersham). The probe used was an 800 base pair *EcoRI/BamHI* insert from pSPT/PGK kindly provided

SUMMARY OF RESULTS

Subject investigated	X-inactivation pattern in T cells	Carrier status for X-linked SCID	Comment
<i>X-linked</i>			
Pedigree 1			
Obligate carrier	Non-random	Carrier	..
Daughter at low risk with linked probe	Random	Non-carrier	..
Pedigree 2			
Obligate carrier	Non-random	Carrier	..
Daughter at risk, uninformative with linked probe	Random	Non-carrier	..
Pedigree 3			
Daughter of obligate carrier, uninformative with linked probe	Random	Non-carrier	..
<i>Unclear inheritance</i>			
Pedigree 4			
Female with two affected sons	Non-random	Carrier	X-linked inheritance
Sister	Non-random	Carrier	
Sister	Random	Non-carrier	
Pedigree 5			
Female with two affected sons	Random	Non-carrier	Autosomal recessive inheritance
Pedigree 6			
Female with sporadic affected male	Non-random	Carrier	X-linked inheritance

by Fearon and Vogelstein. Before hybridisation the probe was preannealed to sonicated salmon sperm DNA (concentration 1 mg/ml) by boiling for 5 min in 3 mol/l NaCl, 0.4 mol/l sodium citrate, and then incubating at 65°C for 30 min. Filters were washed at a final concentration of 15 mmol/l NaCl, 1.5 mmol/l sodium citrate, 0.1% sodium dodecyl sulphate at 65°C, and then exposed to XAR-5 film at -70°C for 10 days.

In addition all the family members in the X-linked pedigrees were investigated with the *PstI* polymorphism of the probe DXS159 as previously described.

Results (Table)

Carrier Detection in X-linked Pedigrees

The linked probe DXS159 was helpful only in the first of the three X-linked pedigrees. In pedigree 1, subject II₄ is at low risk of being a carrier because she had inherited the same allele as her normal brother and the opposite allele from her carrier sister. The second family was not informative with the probe, and key members of the third family had died, so linked probes were not helpful.

Methylation analysis of the T-cell DNA from the obligate carrier, I₂, in pedigree 1 showed the PGK polymorphism (fig 1, lane 1a) and loss of the 0.9 kb band after the addition of *HpaII* (lane 1b). When DNA from her daughter, predicted with the linked probe to be at low risk, was analysed both bands were still present after the addition of *HpaII* (fig 7 lanes 2a and 2b), hence there is a random active X population in her T cells, which is in accordance with the linked-probe data and which confirms that she is not a carrier.

The obligate carrier from pedigree 2 had a non-random pattern. The two daughters of obligate carriers in pedigrees 2 and 3 had random X populations in their T lymphocytes and are therefore not carriers.

Testing for X-linkage

In families 4 and 5 it is not clear from the pedigree whether the inheritance is autosomal or X-linked.

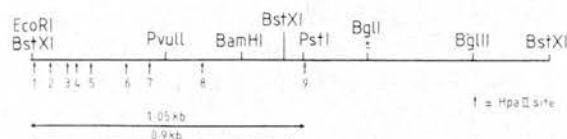


Fig 1—Restriction map of the 5' region of the PGK gene.

The *BstXI* and *BglII* sites are polymorphic. DNA digested with *BstXI* and *PstI* in a heterozygote gives bands of 1.05 kb and 0.9 kb. † indicates *HpaII* site.

60% of all cases must be X-linked.¹ Since the obligate carrier females in X-linked pedigrees have no immunological abnormalities, it has often not been possible to differentiate between X-linked and autosomal inheritance.

The X-linked form has been mapped recently to Xq11-q13 and carrier detection may be possible by use of the probe DXS159 ($\zeta = 5.27$ at $\theta = 0.00$) (heterozygosity = 0.37).² This is useful, but only in families with a clearly X-linked pedigree and either affected or unaffected males to give linkage phase.

Another approach to carrier detection for X-linked disease is the investigation of X inactivation in the cell population affected by the primary defect. By exploiting the differences in DNA methylation patterns between the active and inactive X chromosome,³ Vogelstein et al demonstrated that obligate carriers of X-linked hypogammaglobulinaemia have non-random X inactivation in their B cells.⁴ In the same way obligate carriers of Wiskott-Aldrich syndrome have been found to have non-random X-inactivation in their T-cells.⁵ We have applied the same technique to SCID and have used it to differentiate between autosomal recessive and X-linked inheritance of the disease and to investigate mothers of sporadic male cases.

Subjects and Methods

Principle of the Method

The Lyon hypothesis states that permanent inactivation of one of the two X chromosomes occurs at random in every somatic cell in the female early in embryogenesis.⁶ The pattern of X chromosome inactivation is transmitted in stable fashion to all progeny cells. If one considers a zygote in which one of the X chromosomes carries a mutant gene, initially the normal X will be active in approximately half of the cells and the mutant X active in half of the cells in all the cell populations in the embryo. However, in cells requiring the mutant gene for development, cells with the normal X active will have a selective advantage, so although the initial inactivation was random, the mature cell population will show a non-random pattern of X inactivation.

The method developed by Vogelstein³ takes advantage of the fact that some genes on the X chromosome have different methylation patterns on the active and inactive X, and that this methylation



Fig 2—Analysis of X chromosome inactivation in a normal control.

The fragments indicated by the → are the 1.05/0.9 kb polymorphism of PGK. In the first track the DNA has been digested with *PstI* and *BstXI* but no *HpaII*. In the second track the DNA has been digested with *PstI*, *BstXI* and *HpaII*.

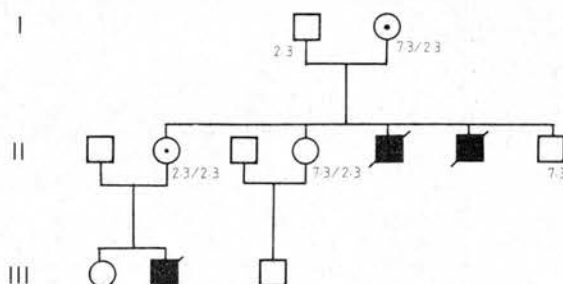


Fig 3—Pedigree family 1.

DXS159 has a *PstI* polymorphism with alleles 7.3 kb and 2.3 kb. The polymorphic bands are marked on the pedigree.

pattern is transmitted in a stable form. Probes from the 5' end of the hypoxanthine phosphoribosyltransferase (HPRT) and phosphoglycerate kinase (PGK) genes which will detect both a polymorphism and also a difference in methylation between the active and inactive X chromosome are used. One endonuclease is used to demonstrate the polymorphism and a methylation-sensitive endonuclease to demonstrate the methylation pattern. The probes used are not themselves linked to the disease but are used simply to demonstrate the X-inactivation pattern in the cell population of interest. The recognition site of *HpaII* is CCGG but *HpaII* only digests when the 5'CG3' dinucleotide is unmethylated. The first eight *HpaII* sites shown in figure 1 are methylated on the inactive X chromosome and unmethylated on the active X chromosome; the ninth *HpaII* site is unmethylated on the active X but is also unmethylated on 20–40% of inactive X chromosomes. The first eight *HpaII* sites and the polymorphic *BstXI* site can be conveniently bracketed by digesting with *PstI* and *BstXI*. This excludes the ninth *HpaII* site and so avoids difficulties in interpreting results which it could cause. *HpaII* therefore cuts *BstXI/PstI*-digested DNA from this region of active X chromosomes into small fragments. *HpaII* does not cut any of the sites on this region of the inactive X chromosome and so the polymorphic fragment sizes are unchanged at 1.05 or 0.9 kb. Figure 2 shows T lymphocyte DNA from a heterozygous normal control. There are two bands present before and after the addition of *HpaII* demonstrating a random active X population in these cells.

Subjects

17 females from 6 families were tested for methylation-sensitive restriction fragment length polymorphisms (RFLPs) for the HPRT and PGK probes. 10 females from 6 separate families were found to be heterozygous for the PGK polymorphism and were investigated further. None of the women were heterozygous for the HPRT polymorphism. X-inactivation in the T lymphocytes of the 10 females heterozygous for PGK was investigated.

In pedigree 1 (fig 3) an obligate carrier, I₂, and her at-risk daughter, II₄, were investigated. An obligate carrier and her daughter were investigated from pedigree 2. From pedigree 3 another daughter of an obligate carrier was investigated.

In pedigree 4 (fig 4) proband II₂ had had two sons who had died from SCID and two brothers who had died in infancy, but unfortunately there was no clinical information about them and it was therefore not clear whether this was the X-linked form of the disease. In this pedigree the proband and her sisters II₄ and II₆ have

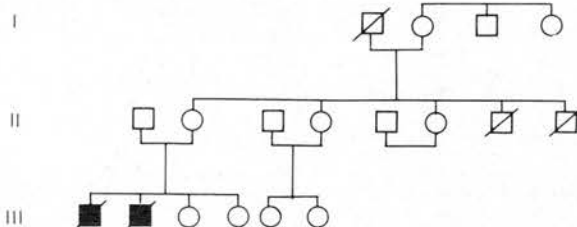


Fig 4—Pedigree family 4.

while in hospital, and only 3.4% of such patients suffered a morbid cardiovascular event. Thus decisions about transfusion may need to take into account both the preoperative haemoglobin level and the expected amount of blood loss during surgery—the latter depending on the surgical procedure, the surgeon, and the technique used.

The increase in mortality we found when haemoglobin levels fell below 8 g/dl corresponds to what is known about physiological responses to both anaemia and surgery.¹ Anaemia reduces the oxygen-carrying capacity of the blood, and the cardiac output usually rises when haemoglobin drops acutely to less than 8 g/dl.¹ Thus the cardiac reserve needed to compensate for new increases in oxygen demand or hypoxaemia is diminished. Surgery and anaesthesia can cause hypoxia by several mechanisms. Therefore the risk of tissue hypoxia is enhanced in the anaemic patient undergoing surgery.

These considerations suggest that thresholds for transfusion may need to be modified for patients with underlying disease and those who are subject to additional physiological stresses unrelated to the surgical procedure, such as hypoxaemia or serious infection. Patients in whom the response of cardiac output to stress is already impaired and those in whom cardiac output has already risen in response to a stress occurring before surgery may be more susceptible to the adverse effects of anaemia during surgery.

There is evidence that patients with acute blood loss may tolerate surgery less well than those with chronic compensated anaemia, such as that due to chronic renal failure.¹² Thresholds for transfusion may also need to be modified for patients with other specific types of anaemia.

Anaemia may also be a marker for more severe underlying illness, which may, in turn, increase the surgical risk.¹³ In our study, however, the relation between preoperative haemoglobin and operative blood loss, on the one hand, and postoperative mortality, on the other, were independent of all confounding variables measured.

Our data on potential confounding variables, obtained retrospectively, might not truly reflect the patient's clinical status. However, there is no reason to think that this method of data collection caused any systematic bias. Our patients differed from others only in their refusal to accept blood transfusion. Intraoperative and postoperative management was otherwise identical to that provided for other surgical patients, and blood volume was maintained with standard methods. If only the least ill patients with preoperative haemoglobin levels between 8 and 10 g/dl had been accepted for surgery, the effect of anaemia on mortality would have been underestimated. However, we found no evidence of such selection.

Our study corroborates the widely held belief that the haemoglobin level is an important independent predictor of postoperative death but calls into question the accepted transfusion threshold of 10 g/dl. Our results also suggest that the amount of blood loss expected is an important consideration in deciding whether to transfuse a patient before operation.

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References at foot of next column

USE OF X CHROMOSOME INACTIVATION ANALYSIS TO ESTABLISH CARRIER STATUS FOR X-LINKED SEVERE COMBINED IMMUNODEFICIENCY

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Summary Analysis of X chromosome inactivation in T-lymphocyte DNA from two obligate carriers of X-linked severe combined immunodeficiency showed a non-random pattern. This method was then used to establish carrier status in at-risk females in X-linked pedigrees. It was further used to differentiate between X-linked and autosomal recessive inheritance of the disease when the mode of inheritance was not clear from the pedigree. In addition, a mother of a boy affected by the sporadic form of the disease was found to have non-random X inactivation in her T lymphocytes and she is therefore a carrier of the X-linked disease.

Introduction

SEVERE combined immunodeficiency (SCID) is a heterogeneous group of disorders that can be inherited in X-linked or autosomal recessive forms. The children are born without any cell-mediated or humoral immunity and invariably die of infection within the first 2 years of life unless treated by bone-marrow transplantation. The clinical features do not allow distinction between the different modes of inheritance and, although all children have absent T-cell function, there is considerable variation in numbers of T and B cells. Approximately 25% of cases of the autosomal recessive form are due to deficiency of the purine-degradation enzyme, adenosine deaminase, and this form can be readily identified. For all types of SCID combined, the ratio of males to females affected is approximately 4:1, so

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Linkage of PGK1 to X-linked severe combined immunodeficiency (IMD4) allows predictive testing in families with no surviving male

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Summary. We present a linkage map of DNA probes around the X-linked severe combined immunodeficiency (IMD4) locus at Xq11–13. DXS159 and PGK1 show no cross-overs with the disease locus (Lod 3.01 at $\theta = 0.00$). The order of loci is DXS1–DXS106–(DXS159–PGK1–IMD4)–DXS72–DXYS1. Members of families whose carrier status has been established by X-inactivation patterns were included in the analysis. As the probe (pSPT/PGK), which is used for investigation of X-inactivation patterns, has been shown to be linked to the disease itself, it is possible to assign phase in mothers of sporadic cases who have been shown to be carriers, even when they have no surviving male offspring.

Introduction

Severe combined immunodeficiency (SCID) is a syndrome in which affected infants lack both cellular and humoral immunity; they die in infancy from overwhelming infection if bone marrow transplantation is not successful. There is an X-linked recessive form and several autosomal forms of SCID, which cannot be distinguished on clinical or immunological grounds, although the autosomal form caused by adenosine deaminase deficiency can be diagnosed by enzyme assay. Female carriers of the X-linked form are immunologically normal but have a non-random pattern of X-inactivation in their T cells (Puck et al. 1987; Goodship et al. 1988). The X-inactivation pattern can be investigated in women who are heterozygous for restriction fragment length polymorphisms detected by probes from the 5' end of the phosphoglycerate kinase and hypoxanthine-guanine-phosphoribosyl transferase genes (Vogelstein et al. 1987). There are differences in the methylation pattern on the active and inactive X chromosome at the 5' end of these genes, differences that are transmitted from cell to cell in a stable fashion and that can be demonstrated using the methylation-sensitive endonuclease *HpaII*.

IMD4 has been mapped to Xq11–q13 (de Saint Basile et al. 1987). A maximum Lod score of 5.27 at $\theta = 0$ has been reported for DXS159 and recombinants have been observed between IMD4 and DXS1, and between IMD4 and DXYS1 (de Saint Basile et al. 1987). Three additional probes in the region, pSPT/PGK, cpX203 and pX65H7, detecting the loci PGK1, DXS106 and DXS72, respectively, have been included in this linkage study in order to localise the disease more precisely.

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Materials and methods

Families

Five families were investigated (Fig. 1). Families KHA, TEB and BER were included in our previous report (de Saint Basile et al. 1987). At least one male in each family had been investigated and fulfilled the diagnostic criteria set out by the WHO Committee on immunodeficiency (WHO Report 1986). In four families, there was unequivocal X-linked transmission on pedigree grounds and, in the fifth family, there was a non-random pattern of X-inactivation in the mother of two affected males. The carrier status of females who had not had affected offspring and who were heterozygous for the pSPT/PGK polymorphism was determined by methylation analysis (Goodship et al. 1988). Eleven females were obligate carriers on pedigree, and the carrier status of an additional seven women was determined by methylation analysis.

DNA analysis

The probes p8 (DXS1), cpX289¹ (DXS159), pSPT/PGK (PGK1), cpX203 (DXS106), pX65H7 (DXS72) and pDP34 (DXYS1) were used to detect restriction fragment length polymorphisms (Davies et al. 1987). DNA extraction, restriction endonuclease digestion, electrophoresis, blotting and hybridisation were carried out using standard procedures (Old 1986). Fibroblasts from two affected males who had had bone marrow transplants were cultured for DNA extraction.

Data analysis

Data were prepared for analysis using LINKSYS (Attwood and Bryant 1988). Two point linkage analysis was carried out using LIPED. The data were also analysed using the LINKMAP section of LINKAGE version 4.7.

The loci used in the LINKMAP analysis were DXS106, DXS159, PGK1 and DXYS1, detected by the probes cpX203, cpX289, pSPT/PGK and pDP34, respectively. No figure is available for the distance between DXS106 and DXS159, and an arbitrary figure of 2 cM was used for the analysis. The dis-

¹The probe used to detect this polymorphism was originally described as cpX73 (DXS159) (Arveiler et al. 1987). However the polymorphism was detected by cpX289 (DXS162) and cpX73 does not detect a polymorphism. It was agreed at the HGM9 meeting in Paris that the probe detecting the *PstI* polymorphism, cpX289, should now be called DXS159

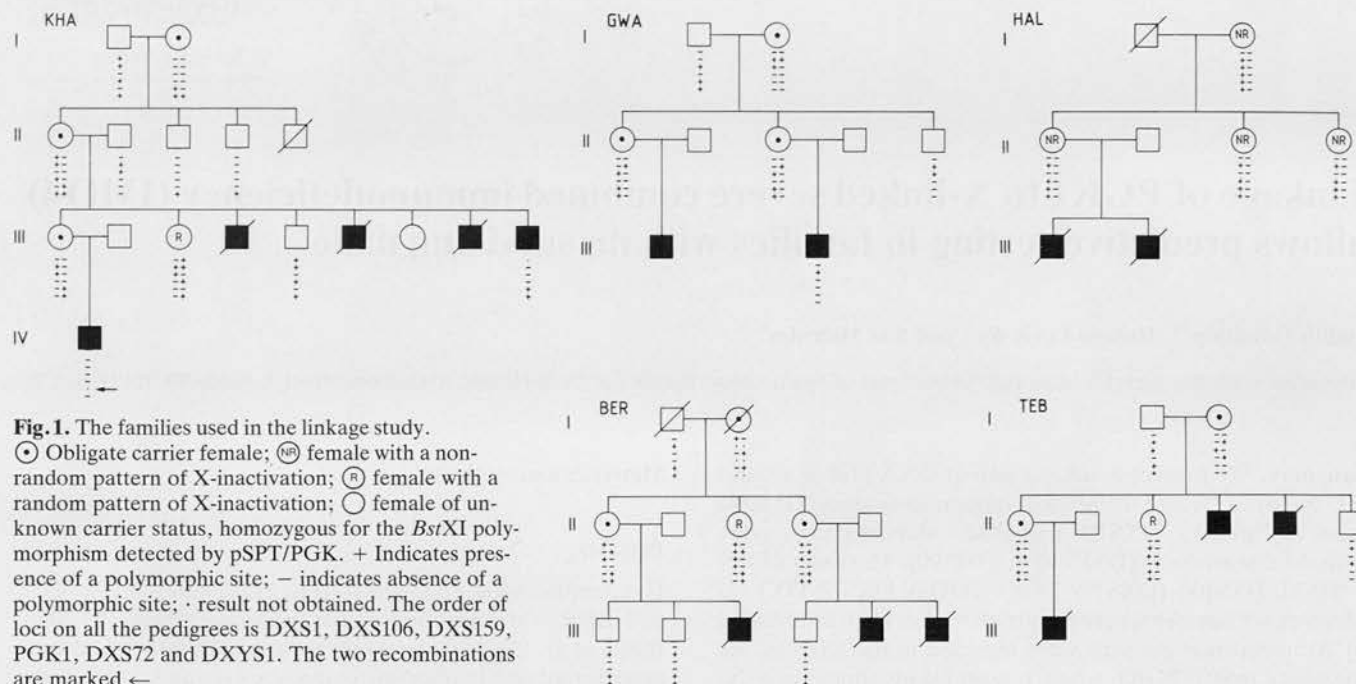


Fig. 1. The families used in the linkage study. \odot Obligate carrier female; \odot female with a non-random pattern of X-inactivation; \odot female with a random pattern of X-inactivation; \circ female of unknown carrier status, homozygous for the *Bst*XI polymorphism detected by pSPT/PGK. + Indicates presence of a polymorphic site; - indicates absence of a polymorphic site; . result not obtained. The order of loci on all the pedigrees is DXS1, DXS106, DXS159, PGK1, DXS72 and DXYS1. The two recombinations are marked \leftarrow

tance estimated between the probes DXS159 and DXYS1 is 8 cM (Arveiler et al. 1987). A recent report gave a DXS159-PGK1 distance of 10 cM with confidence limits ranging from 4 to 19 (Mahtani and Willard 1988). We have not observed any recombinations between DXS159 and PGK1. There is no published estimate of the distance between the probes PGK1 and DXYS1. In view of this discrepancy, three analyses were carried out first using recombination fractions 0.02 0.01 0.07, then 0.02 0.04 0.04 and then using 0.02 0.07 0.01, so moving PGK1 from DXS159 to DXYS1.

Results

The haplotypes are shown on the pedigrees in Fig. 1. The order of loci on which the haplotypes are based is DXS1-DXS106-DXS159-PGK1-DXS72-DXYS1. The haplotype for I_2 of pedigree HAL is ambiguous but there are no crossovers between the five informative probes in any of the possible haplotypes.

There were no recombinations between IMD4 and DXS159, PGK1 or DXS72. The maximum Lod score by two point analysis for IMD4-PGK1, including females whose carrier state was determined by methylation analysis, was 3.01 at $\theta = 0$. Adding the new information from these pedigrees for DXS159-

IMD4 linkage to our previously published data (de Saint Basile et al. 1987) increases the two point Lod score to a max of 7.08 at $\theta = 0$.

Only two crossovers were observed within any of the haplotypes. These were between DXS159 and DXS106 in II_3 of pedigree TEB and between PGK1 and DXYS1 in IV_1 of pedigree KHA (Fig. 1).

LINKMAP analysis of IMD4 against the data from these five pedigrees for the set of markers that flank the gene, i.e. cpX203, cpX289, pSPT/PGK, and pDP34, is presented in Table 1. The maximum Lod scores using LINKMAP become 5.11 at the cpX289 locus and 5.03 at the pSPT/PGK locus in the first analysis (Fig. 2), falling to 4.7 at the pSPT/PGK locus in the second analysis, and 4 at the pSPT/PGK locus in the third analysis with pSPT/PGK placed 7 cM from cpX289 and only 1 cM from pDP34.

PGK1 linkage and X-inactivation analysis for family LAW is presented in Fig. 3a. There was a single affected boy in the family but no material for DNA analysis was available; it was therefore impossible to test which pSPT/PGK or cpX289 allele he had inherited. X-inactivation studies (Fig. 3b) show that his mother has a non-random pattern of active X chromosomes in her T cells and that the active, i.e. normal, X chromosome carries the lower 0.9 kb allele; this is the X chromosome inherited from her father. We are therefore able to predict that the defective gene is carried on the chromosome with the upper 1.05 kb band; in view of the close linkage of IMD4 and PGK1, prenatal diagnosis could be offered in a future pregnancy.

Table 1. Lod scores for X linked immunodeficiency between DXS106 and DXYS1 generated by LINKMAP. In analysis 1 the distances between the four loci were 0.02:0.01:0.07, in analysis 2 the distances were 0.02:0.04:0.04 and in analysis 3 the distances were 0.02:0.07:0.01

	DXS106	DXS159	PGK1	DXYS1
1	- ∞	5.11	5.03	- ∞
2	- ∞	5.11	4.70	- ∞
3	- ∞	5.11	4.00	- ∞

Discussion

IMD4 has been previously mapped to Xq11-13 by its close linkage to DXS159 (de Saint Basile et al. 1987). The order of other probes in the region has been established by a number of studies using somatic cell hybrids, X chromosome deletions

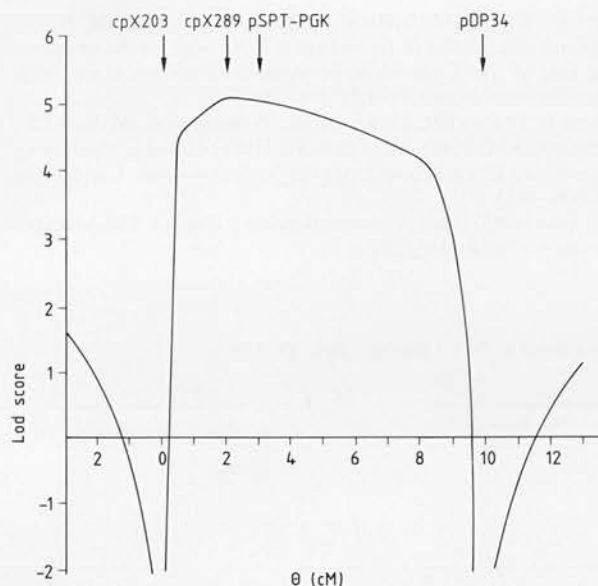


Fig. 2. Lod scores calculated by LINKMAP for varying locations for IMD4 on proximal Xq

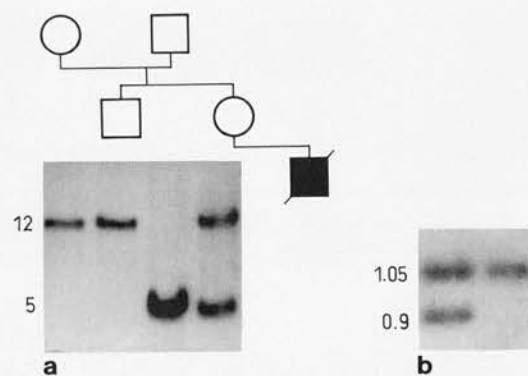


Fig. 3. a Southern blot analysis of family LAW showing the alleles detected by pSPT/PGK after DNA digestion with *Bgl*II. This polymorphism is in linkage disequilibrium with the *Bst*XI polymorphism. The allele sizes are marked in kb. b X-inactivation analysis of proband in family LAW. The DNA in the first track has been digested with *Pst*I and *Bst*XI, and shows the polymorphism. The DNA in the second track has been digested with *Pst*I, *Bst*XI and *Hpa*II. The allele sizes are marked in kb

and duplications, and is known to be centromere-p8-cpX203-cpX289-pSPT/PGK-pX65H7-pDP34 (Cremers et al. 1987; Arveiler et al. 1987; Mahtani and Willard 1988; Cremers et al. 1988). Recombinations have previously been reported between IMD4 and DXS1, and IMD4 and DXYS1 (de Saint Basile et al. 1987). From the recombination observed in family TEB, IMD4 is distal to DXS106. In addition, two families have been reported in which there were deletions on the X chromosome in males with choroideremia; these deletions extended from the XY homologous region and included the probe pX65H7 (Schwartz et al. 1988; Nussbaum et al. 1987). As the males with these deletions did not have SCID, the disease locus must lie proximal to DXS72. Thus, DXS106 and DXS72 are closer flanking markers for the disease locus, and the order is cen-DXS1-DXS106-(DXS159-PGK1-IMD4)-DXS72-DXYS1.

Despite two extensive linkage studies (Arveiler et al. 1987; Mahtani and Willard 1988), the distances between the probes remains uncertain. This has caused difficulties in using LINK-MAP. However, the maximum location score found at pSPT/PGK varies comparatively little within the range of possible distances.

The linkage data for PGK1 and IMD4 and the physical mapping information placing PGK1 between DXS159 and DXS72 provides an additional polymorphic marker that can be used for predictive testing. Using the probes cpX289 and pSPT/PGK, 65% of females should now be heterozygous for closely linked probes.

Furthermore, pSPT/PGK is one of the probes that can be used for investigation of X-inactivation patterns in carrier testing. As we have shown that PGK1 is linked to IMD4, we can assign phase in a female without following the tracking procedure normally used. It is now possible to assign phase in families that do not have an adequate family structure for tracking the linked probe through the family. In addition, it is possible to assign phase in mothers of sporadic males, mothers who are shown by inactivation studies to be carriers even if their affected child has died before tissue could be stored for DNA extraction and although they may themselves carry a new mutation. This ability to assign phase in a woman without looking at the mutation directly or tracking haplotypes is a novel situation.

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Close linkage of random DNA fragments from Xq 21.3-22 to X-linked agammaglobulinaemia (XLA)

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Summary. Linkage analysis of 15 families affected by X-linked agammaglobulinaemia (XLA) showed close linkage with three probes located towards the centre of the long arm of the X chromosome. No cross-overs were found using pXG12 (DXS94) lod 6.6 or S21 (DXS17) lod 4.4. One cross-over was found with 19.2 (DXS3). This confirms and extends a previous linkage study (Kwan et al. 1986) which demonstrated linkage with S21 and 19.2. Of the families 14 were informative for either pXG12 or S21 and these probes should thus be of great diagnostic value. No evidence of heterogeneity was found in the XLA families but several cross-overs within this region were detected in a family with the X-linked hyper-IgM syndrome confirming this disease as a separate clinical entity.

Introduction

X-linked agammaglobulinaemia (XLA) is a severe humoral immunodeficiency in which affected males lack all immunoglobulin isotypes. Prior to treatment with antibiotics and regular immunoglobulin therapy, the children died of infections in early childhood (Rosen et al. 1984). Since the majority of affected children have pre-B cells in their bone marrow (Burrows et al. 1979) but lack circulating mature B cells, the mutated gene responsible for the disease is presumably involved in B cell differentiation. After the age of 6-9 months, once maternal acquired IgG has diminished, affected males present clinically with repeated bacterial infections. The diagnosis is readily made by the hypo- or agammaglobulinaemia involving all isotypes, the lack of circulating B cells and consequent lack of plasma cells. Obligate carrier females are not detectable by any immunological criteria. Although B cells can be detected prior to 20 weeks gestation in foetal blood (Linch et al. 1982) this may not be sufficiently reliable to offer accurate prenatal diagnosis especially as some heterogeneity with respect to the presence or absence of B cells in XLA patients has been described (Leickley and Buckley 1986). With the advent of DNA probes that loosely mapped the XLA locus (Kwan et al. 1986; Mensink et al. 1986) carrier detection and prenatal diagnosis by analysis of chorionic villus biopsy material became

feasible. We now report that a further probe pXG12 together with probe S21 closely maps the XLA locus to the Xq 21.3-22 region of the X chromosome.

Materials and methods

Sample collection

Samples were collected from members of 15 families in each of which an affected boy with X-linked agammaglobulinaemia had been a patient at either the Hospital for Sick Children, London, East Birmingham Hospital, Northwick Park Hospital, London or Hôpital d'Enfants Malades, Paris. Fifteen index patients were diagnosed as having XLA with characteristic immune profiles. The index patient in a 16th family was diagnosed as having the hyper-IgM syndrome; he had abnormally high serum IgM levels, no serum IgG or IgA and his B cells present in normal numbers had only IgM and IgD on the cell surface. Women were only considered to be carriers if they had at least two affected sons or an affected son plus an affected maternal uncle or cousin.

Blood samples were collected in ethylenediaminetetraacetate (EDTA). One patient (MB) suffered from choroideraemia and mild mental retardation and was shown by cytogenetics and DNA analysis to have an Xq deletion (Robertson et al. 1986).

X-chromosome probes

Probes PXXG12 (DXS94), S21 (DXS17) and 19.2 (DXS3) were used to detect restriction fragment length polymorphisms. PXXG12 detects a *Pst*I polymorphism with alleles 7.2 kb and 6.5 kb; S21 a *Taq*I polymorphism with alleles 2.2 kb and 2.0 kb; 19.2 a *Taq*I polymorphism with alleles of 5.0 kb or a doublet of 2.9 and 2.2 kb.

Detection of polymorphisms

DNA extraction, restriction enzyme digestion, electrophoresis, blotting and hybridisation were carried out by standard procedures (Old 1986). Lod scores were calculated using the LIPED program run on an IBM PC.

Results

Mapping of PXG12

pXG12 had previously been mapped to the proximal region of the long arm of the X chromosome (Goodfellow et al. 1985). Two lines of evidence show that it lies between 19.2 and S21. DNA was extracted from a man, MB, suffering from choroideraemia and mild mental retardation but in whom there was no impairment of either cellular or humoral immune function. High resolution chromosome studies showed that band Xq21 was about half its normal size, probably due to a deletion of sub-band Xq21.1 (Hodgson et al. 1987). DNA studies showed that both the X locus of DXYS1 (pDP34) and DXS3 (19.2) were deleted. However, a clear 6.5 kb allele of PXG12 was observed, showing that PXG12 lies outside the DXYS1-DXS3 segment. Evidence that it falls below 19.2 rather than above pDP34 comes from multi-point analyses in families. In one family with individuals heterozygous for all four probes the pattern of inheritance could most simply be explained (i.e. only assuming single cross-overs) if the order is pDP34, 19.2, PXG12, S21 (Arveiler and Saint Basile, in preparation).

Table 1. Summary of lod scores between XLA and DXS17 and DXS94, both of which map to Xq21.3 to Xq22

Locus	DXS17	DXS94	DXS3
Probe	S21	pXG12	19.2
Location	Xq21.3-22	Xq21.3-22	Xq21.3-22
Recombination fraction	Lod scores		
0			
0.00	4.44	6.65	—
0.01	4.43	6.64	2.40
0.05	3.97	5.97	3.63
0.10	3.48	5.27	3.41
0.15	2.98	4.56	3.07
0.20	2.45	3.82	2.65
0.25	1.93	3.07	2.19
0.30	1.42	2.33	1.71
0.35	0.93	1.61	1.24
0.40	0.51	1.06	0.78
0.45	0.18	0.39	0.32
0.45	0.00	0.00	0.00
Combined data this study and Kwan et al. (1986)			
0.00	6.61	—	—
0.05	—	—	7.28

Linkage of S21, pXG12 and 19.2 in families with XLA

Members of 15 XLA families were analysed for restriction fragment length polymorphism (RFLPs) using the probes pXG12, S21 and 19.2. No cross-overs were found using either S21 or pXG12 and lod scores of 4.44 and 6.65 respectively were calculated (see Table 1). Combining this data for S21 with that published by Kwan et al. (1986), a lod score of 6.61 at $\theta = 0$ is found. One cross-over was found with the probe 19.2 but this was in the only family uninformative for both pXG12 and S21. Therefore using the two probes S21 and pXG12 it is possible to improve considerably carrier detection for female relatives at risk.

Linkage studies in a family with X-linked hyper-IgM syndrome

Analysis for RFLPs was carried out in a family with X-linked hyper-IgM syndrome (Fig. 1). One cross-over with known phase was observed with probes 19.2 and pXG12 as pedigree individual III₂ carries the same haplotype as his affected brother: S21 was uninformative in this case. A further cross-over with S21 was observed in a different part of the pedigree. The normal brother (II₂) has inherited the same haplotype as his two carrier sisters. As I₁ is also informative for S21 the cross-over in this case does not lie between pXG12 and S21. The results from this family indicate that X-linked hyper-IgM syndrome and XLA are not allelic.

Discussion

There are several X-linked immunodeficiency syndromes that can result in hypo- or agammaglobulinaemia. These are XLA, X-linked hyper-IgM syndrome, Wiskott-Aldrich syndrome, X-linked lymphoproliferative syndrome where a minority of cases present hypogammaglobulinaemia, and X-linked severe combined immunodeficiency where both cellular and humoral immunity are lacking. The genetic defects in these syndromes although resulting to some extent in hypogammaglobulinaemia probably comprise distinct gene mutations. Evidence in favour of this comes from the work of Conley et al. (1986) who showed that the mutation in XLA was specific to B cells whereas that in X-linked severe combined immunodeficiency affected both T and B cells (Puck et al. 1986).

Although it is usually possible to differentiate between all these syndromes on the basis of immunity function testing, some heterogeneity has been described in families with XLA with respect to the presence or absence of B cells (Leickley and Buckley 1986). Because of this, it is important to have as homogeneous a study population as possible in order to con-

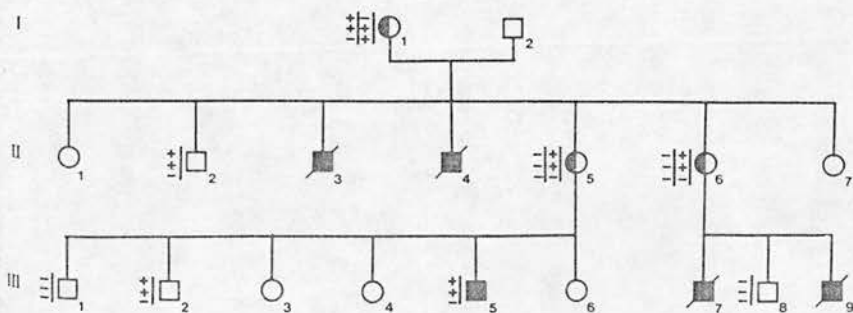


Fig. 1. Pedigree with RFLP data of family with X-linked hyper-IgM syndrome. Results are given for 19.2 (top) pXG12 (centre) S21 (bottom); (+) indicates presence of restriction site; (—) indicates absence of restriction site. Recombinations have occurred between the disease locus and all the probes represented in the haplotype in the meioses leading to II₂ and III₂.

firm genetic linkage. The diagnostic criteria for XLA were the lack of immunoglobulins of all isotypes and <1% circulating B cells in the peripheral blood. All of the families studied had clear-cut X-linked pedigrees.

Initial indication of the chromosomal localisation of XLA came from Kwan et al. (1986) who found linkage with 19.2 $\theta = 0.04$ with a lod score of 3.65 and with S21 at $\theta = 0$ with a lod score of 2.17. Mensink et al. (1986) confirmed linkage with 19.2 by examining a single large family which gave a lod score of 3.30 at a recombination fraction of 0.06. However, examination of a further family suggested to them that there may be genetic heterogeneity with clinically and immunologically indistinguishable forms of the disease mapping to different loci. We have confirmed and extended linkage data with 19.2 and S21 and also found another probe pXG12 which is very closely linked ($\theta = 0$, lod score 6.66). As 14 out of 15 of our families were informative for either S21 or pXG12 and as we have observed no cross-overs with either probe we have found no evidence for any genetic heterogeneity. The 15th family unfortunately was not informative for either S21 or pXG12. In this pedigree one cross-over with unknown phase was observed with probes 19.2 and pDP34.

Nonetheless it is difficult to reconcile the lack of genetic heterogeneity found by our study and that of Kwan et al. (1986), with the clear-cut XLA family described by Mensink et al. (1986) in whom no linkage with the Xq21-22 region was found. The authors claim that the affected males were indistinguishable from others with XLA by immunological criteria. As they found negative lod scores with the markers they examined along all of the X chromosomes, they suggest autosomal dominant and male limited inheritance (Ott et al. 1986). Since the presumed genetic defect in XLA involves the differentiation step from pre-B cell to B cell there should be non-random inactivation of the X chromosome in surviving B cells of carrier females (Conley et al. 1986). If the disease is autosomal such X-inactivation would be random and this could be tested by methylation patterns (Vogelstein et al. 1985). Alternatively Mensink et al. (1987) propose that the heterogeneity found in their family could be due to mosaicism in the maternal grandfather (half chromatid mutation).

In contrast to the XLA families, at least two cross-overs were observed in the X-linked hyper-IgM pedigree. This clearly indicates a distinct localisation for the genetic defect in this disease.

Previous mapping data has placed S21 and 19.2 within bands Xq21.3-22 (Goodfellow et al. 1985). Extensive probe-to-probe linkage data are not available and the best estimate of the distance between 19.2 and S21 is $\theta = 0.06$, based on one cross-over in 17 meioses (Drayna and White 1985). The same study suggested on linkage grounds that 19.2 maps above S21 on Xq. Our overall figures in the XLA families of $\theta = 0.05$ for 19.2 and $\theta = 0$ for S21 agrees well with this map distance.

The combination of deletion data and probe-to-probe data indicates that the gene order is centromere-pDP34-19.2-pXG12-S21. Due to the lack of recombination events in the families we have studied we are unable to place XLA exactly within this cluster although the two cross-overs observed with 19.2 ($\theta = 0.05$) and the fact that 19.2 is deleted in the patient with choroideraemia but normal immunity suggests that the XLA locus must lie very close to S21 and pXG12. Since both probes are highly polymorphic and closely linked to XLA and to each other (B. Arveiler and G. de Sainte Basile; in preparation), they can be used in genetic counselling to greatly im-

prove genetic prediction. Some female relatives can be shown to have a high risk of being a carrier, and coupled with pedigree information others can be given a reassuringly low risk.

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Genetic Prediction in X-Linked Agammaglobulinaemia

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S21 (DXS17) and pXG12 (DXS94), two probes linked to the locus of X-linked agammaglobulinaemia (XLA), were used for genetic prediction in 13 such families. A method of allowing for nonallelic genetic heterogeneity was demonstrated in the calculation of the genetic risks, specifying a certain proportion of unlinked families. We further estimated the impact due to the uncertainty of the proportion of unlinked families on the final genetic risks in each family and this can be taken into account during genetic counselling.

Key words: XLA, nonallelic genetic heterogeneity, genetic linkage, gene tracking

INTRODUCTION

X-linked agammaglobulinaemia (XLA), first described by Bruton [1952], is characterised by panhypogammaglobulinaemia, virtual absence of B lymphocytes in blood, and normal number of pre-B cells in bone marrow [Pearl et al., 1978; Rosen et al., 1983; Conley, 1985]. It was always fatal until the use of antibiotics and immunoglobulin replacement. Even with treatment, long-term survivors may suffer chronic lung disease and are susceptible to severe disseminated viral infections [Lederman and Winkelstein, 1985]. The female carriers of this X-linked recessive disease are healthy and cannot be identified immunologically. However, carrier status can be ascertained by analysing X-chromosome inactivation in the B-cell lineage [Fearon et al., 1987], but this is not easy in practice. Since the XLA gene locus has recently been mapped to Xq21.3-22 using linkage to restriction fragment length polymorphism (RFLP) [Kwan et al., 1986; Mensink

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et al., 1986; Malcolm et al., 1987], it is now possible to offer carrier detection and prenatal diagnosis using gene tracking. However, the question of nonallelic genetic heterogeneity [Mensink et al., 1986; Mensink et al., 1987a; Ott et al., 1986] has complicated this approach in genetic counselling.

Here we demonstrate that the closely linked probes pXG12 and S21 are clinically useful in most families and demonstrate a method of allowing for nonallelic genetic heterogeneity in the risk calculation.

MATERIALS AND METHODS

Families

All index patients satisfied the diagnostic criteria of the WHO scientific group on immunodeficiency [Rosen et al., 1983]. Out of 13 XLA families, 11 were studied previously and demonstrated linkage between the XLA locus and probes pXG12 (DXS94), S21 (DXS17), and 19-2 (DXS3) [Malcolm et al., 1987]. One of the other 2 families has only one affected male living, with an affected brother who died. One family contains a single affected male with the mother wanting prenatal exclusion. Seventeen obligate carriers and 34 females at risk of being carriers were studied.

We also include one additional family previously reported in 1962 [Jamieson and Kerr, 1962] as XLA, which has been studied since the original linkage analysis [Malcolm et al., 1987]. The only living affected male was investigated by Dr. Roifman in Toronto recently and was found to have no peripheral B cells and panhypogammaglobulinaemia.

DNA Analysis

DNA extraction from blood and chorionic villi, restriction enzyme digestion, electrophoresis, Southern blotting, and hybridisation were performed by standard methods [Old, 1986]. X-chromosome specific probes pXG12 (DXS94), S21 (DXS17), and 19-2 (DXS3) [Drayna et al., 1984; Aldridge et al., 1984; Goodfellow et al., 1985] were used in the initial linkage analysis [Malcolm et al., 1987], but only pXG12 and S21 were used in the genetic counselling of potential carriers and prenatal prediction.

Risk Calculations

Proportion of families unlinked to S21/pXG12. There are now 27 XLA families published [Kwan et al., 1986; Malcolm et al., 1987; Ott et al., 1986] which are informative for linkage analysis with S21 and/or pXG12. Two families have significantly negative lod scores in relation to the S21 locus using multipoint linkage analysis and thus appear not to be linked to the S21 locus [Ott et al., 1986], but there are no other crossovers with S21 or pXG12 in the remaining 25 families. One family (pedigree Z) definitely demonstrates nonallelic genetic heterogeneity [Mensink et al., 1986; Ott et al., 1986], but the other (pedigree EsB) depends on the assumption that one boy (III-1) who died in childhood before 1950 was affected with XLA. Another such family is the additional family previously reported by Jamieson and Kerr [1962] as XLA and in whom the only surviving affected male was found to have no peripheral B cells and panhypogammaglobulinaemia, which are the immunological features of XLA. Our recent linkage analysis shows 2 crossovers between S21 and the disease locus out of 3 informative meioses. On the present evidence the proportion of XLA families unlinked to the region of Xq21.3-22 is likely to be about 10% and unlikely to exceed 30%.

Method of allowing for nonallelic genetic heterogeneity. The published families with S21 linkage data [Kwan et al., 1986; Mensink et al., 1987a; Malcolm et al., 1987], excluding the 3 unlinked families, give a maximum lod score of 10.5 at a recombination fraction (θ) of 0 with 90% confidence intervals 0 to 0.04 as defined by taking the range of θ at one less than the maximum lod score [Conneally et al., 1985]. This represents 40 informative meioses observed in 15 families with no recombination between the disease locus and S21. Twenty-two informative meioses were observed with no recombination with pXG12 in 11 families in our original study [Malcolm et al., 1987]. Therefore, we decided to use S21 and pXG12 in genetic counselling of XLA families, quoting a recombination fraction of 0.04.

As most pedigrees are small, significant lod scores are unlikely to be observed within any one family. However, the larger the family and the greater the number of informative meioses, the greater is the certainty that the disease locus in that family is linked to S21/pXG12. We have developed a method of calculation that allows for nonallelic genetic heterogeneity which incorporates a specified proportion of unlinked families as well as the probability of linkage within that particular family to the DNA markers used for genetic prediction. The correct figure for the proportion of unlinked families is uncertain but we show what effect using figures between 10% and 30% have on the final prediction in any particular family. This can be taken into account when counselling the family.

The formal calculations are as follows. For any individual the probability of a specific XLA genotype, given the pedigree and marker (RFLP) data, can be expressed as

$$\Pr(\text{genotype}|\text{pedigree}) = \frac{\Pr(\text{genotype, pedigree})}{\Pr(\text{pedigree})} \quad (1)$$

If there is genetic heterogeneity, the numerator and denominator in this expression will be made up of 2 parts, depending on whether the pedigree is linked or unlinked, for example the numerator will be

$$\begin{aligned} &\Pr(\text{linked}) \cdot \Pr(\text{genotype, pedigree}|\text{linked}) \\ &+ \Pr(\text{unlinked}) \cdot \Pr(\text{genotype, pedigree}|\text{unlinked}) \end{aligned} \quad (2)$$

In the first part of the above expression (Eq. 1), the prior probability that the disease segregating in that pedigree is in fact linked with the marker locus is multiplied by the probability of the specific genotype in the individual in question and the pedigree data given that the disease is linked to the marker ($\theta = 0.04$). In the second part of the expression (Eq. 2), it is assumed that the disease is not linked to the marker (i.e., $\theta = 0.5$). Probability (Eq. 1) can therefore be expressed as

$$\begin{aligned} &\frac{\Pr(\text{linked}) \cdot \Pr(\text{genotype, pedigree}|\text{linked})}{\Pr(\text{linked}) \cdot \Pr(\text{pedigree}|\text{linked})} \\ &+ \frac{\Pr(\text{unlinked}) \cdot \Pr(\text{genotype, pedigree}|\text{unlinked})}{\Pr(\text{unlinked}) \cdot \Pr(\text{pedigree}|\text{unlinked})} \end{aligned} \quad (3)$$

$\Pr(\text{pedigree}|\text{linked})$ is the probability of the pedigree's assuming the XLA genotype of the individual at risk is unknown and given that the disease is linked to the marker.

Family 1

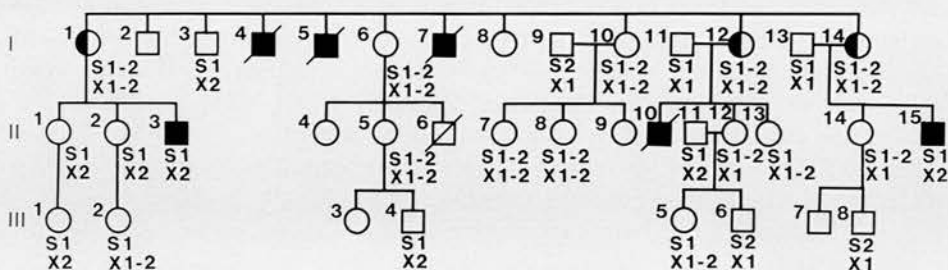


Fig. 1. Pedigrees of the 13 families with S21 (DXS17) Taq 1 polymorphism (S1 = 2.2 kb; S2 = 2.0 kb) and pXG12 (DXS94) Pst 1 polymorphism (X1 = 7.2 kb; X2 = 6.5 kb).

These probabilities, together with their equivalents where the disease is unlinked to the marker, can be obtained from the computer programme Liped [Ott, 1974], in which recent versions give specific instructions for risk calculation.

RESULTS

Carrier Detection

The pedigrees and the segregation of the S21 and/or pXG12 alleles are shown in Figure 1. Table I lists all obligate and potential female carriers from the 13 families, giving risk estimates and the possibility of prenatal diagnosis. The final risks combining pedigree and DNA data are calculated assuming the proportion of unlinked families to be 0%, 10% and 30%. Of the 17 obligate carriers, 7 are under the age of 45 yr and can all be offered prenatal diagnosis, using S21 in 2, pXG12 in 4, and either probe in one. Of the 34 females at risk of being carriers, 17 have their risks increased, 15 decreased and 2 unchanged by the RFLP results. Eleven of the 17 women whose risks were increased are under 45 yr and 7 of them can be offered prenatal prediction.

The Impact of Varying the Proportion of Unlinked Families in Predicting Low Risks

Of the 15 women whose carrier risks were decreased, 3 were already below 5% and 7 were reduced to under 5% if the proportion of unlinked families is assumed to be 10%. Of these seven, 6 would still have a risk below 5% if a figure of 30% unlinked families were used; the other would have a risk of 5.14% (Table I).

Prediction in Males

In family 3, II-1 was shown to be at high risk of being a carrier with both S21 and pXG12. She is heterozygous for S21 and hence prenatal diagnosis is possible. However, she was well into the last trimester of her second pregnancy before the linkage study of XLA was completed. Pregnancy proceeded to term and a male infant (III-2) was born. RFLP analysis of cord blood indicated that he runs a low risk of being affected with XLA. With the family having three meioses supporting linkage with S21, the risk to her son is 4.25–5.62% (assuming 10% or 30% unlinked families, respectively). He was shown to have normal numbers of circulating B lymphocytes and normal immunoglobulin level and is clinically normal at age one yr.

A woman (family 13) with only one son affected with XLA but no family history presented for prenatal exclusion at 9 wk of pregnancy. She was shown to be heterozygous for S21 and the affected son inherited the 2.2-kilobase (kb) allele. Chorionic villus sampling was performed and the fetus was shown to have a 46, XY chromosome constitution and inherited the opposite 2.0-kb allele to the affected boy. A residual risk of 10% of the fetus being affected with XLA was quoted to the parents. This figure takes into account the possibility that the affected boy is a new mutation and that the mother is linkage phase unknown if she were a carrier. The calculated risk was 7.97%, 10.79%, and 13.6% according to whether 10%, 20%, or 30% of families are unlinked. They accepted the risk and a male infant was born at term. Analysis of both cord blood at birth and peripheral blood at age 2 wk showed a normal number of B lymphocytes. As he is still under age 6 months, serum immunoglobulins are mainly of maternal origin.

DISCUSSION

Of the 8 described X-linked immunodeficiency syndromes [Rosen et al., 1983; Densen et al., 1987], 6 have been mapped by RFLP linkage to different loci on the X-chromosome in the last 18 months [Kwan et al., 1986; Baehner et al., 1986; Mensink et al., 1987b; Skare et al., 1987; de Santa Basile et al., 1987; Peacocke and Siminovich, 1987]. For XLA, the presumed defect is a block in the differentiation from pre-B cells to B cells [Pearl et al., 1978] and has been shown to be intrinsic to the B-cell lineage [Fearon et al., 1987]. Until recently female carriers could not be detected, but once linkage to RFLPs was demonstrated it became possible to use gene tracking in genetic prediction [Kwan et al., 1986]. In our study of 34 potential carriers, gene tracking with S21 and pXG12 gave information that could be used in genetic counselling in 32. Of a total of 19 obligate or potential carriers under the age of 45, prenatal prediction using these probes is possible in 15. However, the present sample is biased towards optimum family structure since most were selected for the original linkage study [Malcolm et al., 1987]. The combination of S21 (heterozygosity frequency 0.45) and pXG12 (heterozygosity frequency 0.48) can be expected to render about 70% of females in other families amenable to gene tracking. There is no evidence of linkage disequilibrium between S21 and pXG12. As expected with an X-linked lethal disease, we detected no linkage disequilibrium with XLA and either probe.

There are several potential unrelated sources of error in using linked probes. The problems of recombination, nonpaternity, and new mutation have been discussed previously [Winter et al., 1985; Pembrey, 1986]. Here we have particularly addressed the problem of nonallelic genetic heterogeneity. Until tests independent of linkage analysis can distinguish which gene locus is involved in any particular XLA family, nonallelic genetic heterogeneity must be incorporated into any genetic prediction.

We demonstrated here how one can allow for the problem of nonallelic genetic heterogeneity in risk calculation. However, there is still a degree of uncertainty in risk prediction due to the fact that one cannot be sure about the proportion of unlinked families, whether it is 10%, or perhaps 30%; though it is likely to be about 10% on present evidence. This degree of uncertainty (depending on whether unlinked families are 10% or 30%) is less in a large family (family 1) with linkage data from many members than a smaller family (family 9) with little or no linkage data with the marker.

This difference in the degree of uncertainty can be illustrated by comparing II-12 in family 1 with I-2 in family 9 where the RFLP results favour a new mutation during

TABLE I. Genetic-risks of Female Carriers Using Pedigree Information Alone or With RFLP Results*

Subjects	Pedigree risk (%)	Pedigree + DNA risk (%) when proportion of unlinked families is			Prenatal prediction with S = S21, X = XG12
		0	0.1	0.3	
Family 1					
I-1	100 (O.C.)				>45 years
I-12	100 (O.C.)				>45 years
I-14	100 (O.C.)				>45 years
I-6	27.27	24.17	24.23	24.38	>45 years
I-10	50	50	50	50	>45 years
II-1	50	95.98	95.08	92.71	N.I.
II-2	50	95.98	95.08	92.71	N.I.
II-5	9.09	1.48	1.62	2.01	
II-7	25	48.13	47.69	46.53	S + X
II-8	25	48.13	47.69	46.53	S + X
II-12	33.33	2.05	2.64	4.22	
II-13	50	95.99	95.12	92.8	X
II-14	20	1.04	1.34	2.16	
III-1	25	48	47.57	46.4	N.I.
III-2	25	48	47.57	46.4	X
III-5	16.67	1.03	1.32	2.11	
Family 2					
I-1	100 (O.C.)				>45 years
I-2	100 (O.C.)				>45 years
I-4	33.35	58.15	57.7	56.49	>45 years
II-5	16.68	55.82	55.11	53.21	N.I.
Family 3					
I-2	100 (O.C.)				>45 years
II-1	20	92	89.29	82.55	S
III-2 ^a	16.67	3.69	4.25	5.62	
Family 4					
I-2	27.28	10.32	11.5	14.1	>45 years
II-1	100 (O.C.)				S + X
II-4	9.1	3.46	3.85	4.71	
III-4	4.57	1.75	1.94	2.38	
Family 5					
I-2	33.34	0.02	1.52	5.14	>45 years
II-1	100 (O.C.)				X
Family 6					
I-2	20	63.86	62.7	59.69	>45 years
II-1	100 (O.C.)				X
II-3	10	61.3	59.94	56.43	X
Family 7					
II-1	100 (O.C.)				>45 years
II-2	100 (O.C.)				>45 years
II-3	50	96	94.94	92.16	>45 years
II-4	33.33	92.31	90.95	87.39	>45 years
Family 8					
I-2	50	66.63	65.98	64.4	>45 years
II-1	100 (O.C.)				S
II-2	25	5.15	5.92	7.82	
Family 9					
I-2	42.86	4.16	10.24	20.35	>45 years
II-2	100 (O.C.)				S
II-3	14.3	3.56	5.25	8.05	
III-3	50	88.65	82.58	72.49	S

(continued)

TABLE I. Genetic-risks of Female Carriers Using Pedigree Information Alone or With RFLP Results* (continued)

Subjects	Pedigree risk (%)	Pedigree + DNA risk (%) when proportion of unlinked families is			Prenatal prediction with S = S21, X = XG12
		0	0.1	0.3	
Family 10					
I-2	8.57	0.00	0.29	1.01	
II-2	100 (O.C.)				X
II-6	2.87	0.01	0.11	0.34	
II-7	4.31	0.02	0.17	0.53	
Family 11					
I-2	100 (O.C.)				>45 years
II-1	100 (O.C.)				X
Family 12					
I-2	11.11	36.52	36.23	35.42	>45 years
II-2	100 (O.C.)				>45 years
Family 13					
I-2	66.67	66.67	66.67	66.67	S
II-2 ^b	33.33	5.15	7.97	13.61	

*O.C., obligate carrier; N.I., not informative.

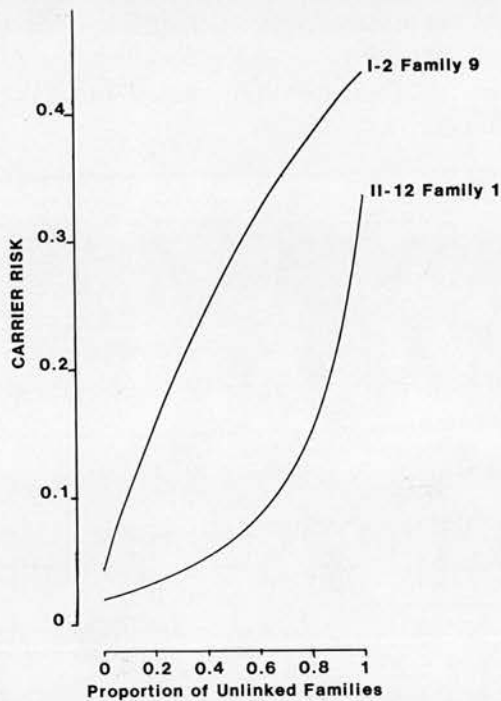
^aNewborn male at risk.^bMale fetus at risk.

Fig. 2. Change of carrier risks with the proportion of unlinked families rising from 0% to 100%.

spermatogenesis in I-1. Both are potential carriers with pedigree risks between 33% and 43% which are reduced to under 5% with the RFLP results if all XLA mutations were linked to S21/pXG12. However, if one assumes 30% of families are unlinked, the risk of I-2 in family 9 will be 20.35% while that of II-12 in family 1 is still 4.22%. The change of these 2 women's carrier risk with the proportion of unlinked families is shown in Figure 2.

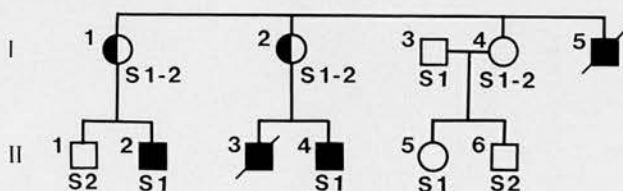
Defining a subgroup of families unlinked to Xq21.3-22 by RFLP analysis will refine our classification of X-linked agammaglobulinaemia and facilitate the discovery of immunological differences between the subgroups.

Another possible explanation for pedigree Z as reported by Mensink et al. [1987a] in which recombinations between disease and S21 occurred can be germline mosaicism. This phenomenon has been convincingly demonstrated in Duchenne muscular dystrophy [Darras and Francke, 1987], but it is difficult to validate this possibility in pedigree Z. Regarding the family which was first reported by Jamieson and Kerr, [1962], germline mosaicism cannot explain the recombinations observed between disease and S21.

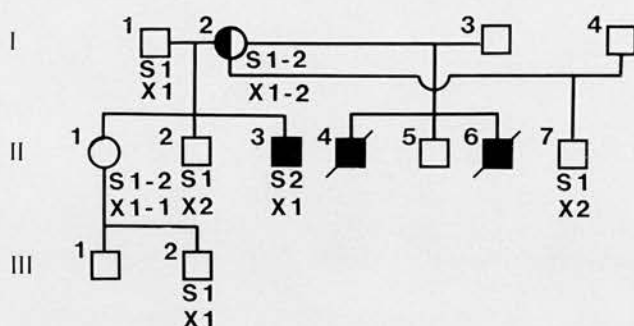
It is also feasible, with the linkage data, to indicate where the mutation first started in a family, e.g., in families 5 and 10, the mutation most likely arose during spermatogenesis in the grandfathers. There are both practical and biological interests in determining whether the mutation rate in males and females is equal with respect to any X-linked disease [Winter, 1980]. However, the immediate practical benefit in pinpointing the mutation in a family is to exclude risks in all relatives except descendants of the carrier of the new mutation.

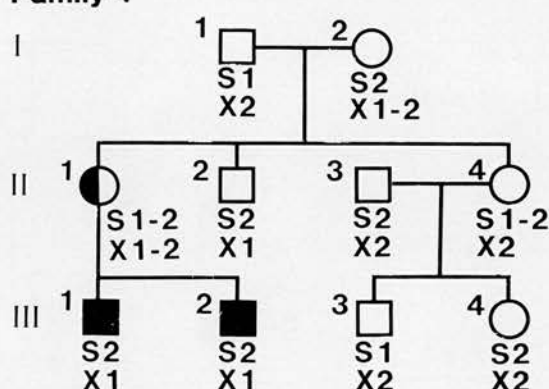
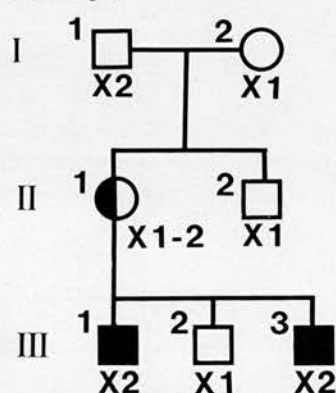
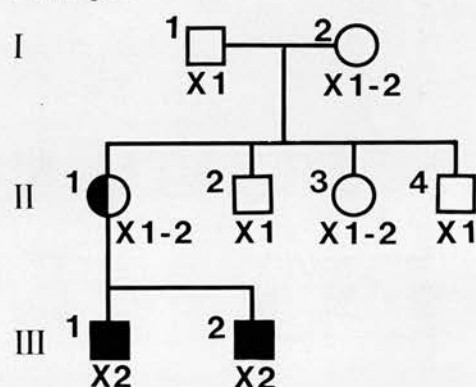
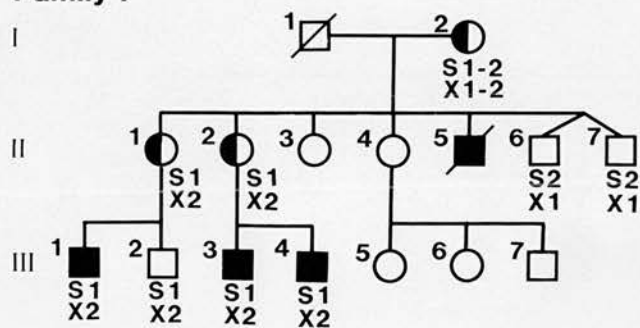
It is our experience when using these linked probes in counselling XLA families that most of the women welcome clarification of their carrier risks despite the limitations and inherent errors of the test. We also mention the availability of prenatal prediction if it is appropriate and possible. The attitudes of potential carriers towards prenatal diagnosis are mixed depending on personal experience of the disease within a family and individual belief. Obviously, the uncertain prognosis of the disease with better treatment is another factor to be considered in genetic counselling.

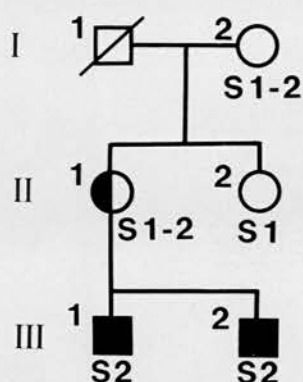
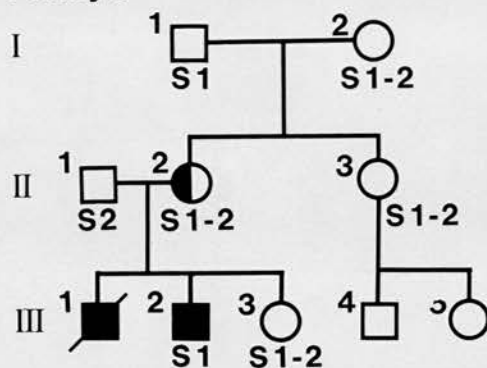
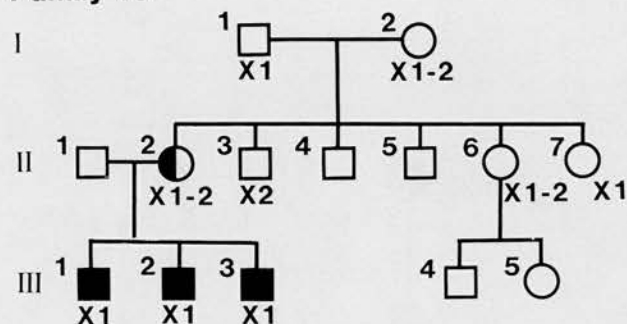
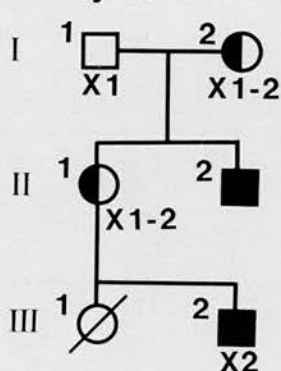
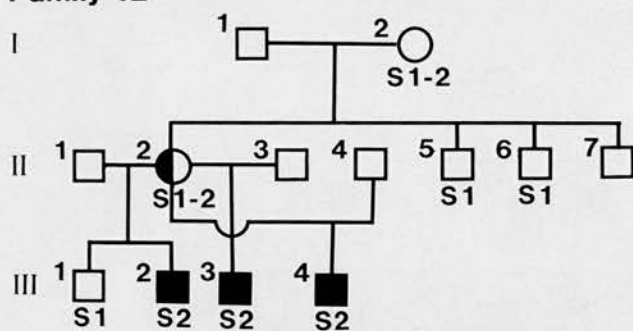
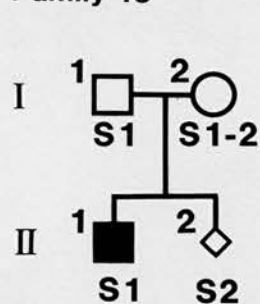
Family 2



Family 3



Family 4**Family 5****Family 6****Family 7**

Family 8**Family 9****Family 10****Family 11****Family 12****Family 13**

ACKNOWLEDGMENTS

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Prenatal diagnosis of X-linked choroideremia with mental retardation, associated with a cytologically detectable X-chromosome deletion

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Summary. We describe a family in which an X-chromosome deletion is segregating with choroideremia, an X-linked recessive condition. The DNA sequences DXYS1 and DXS3, defined by the probes pDP34 and 19.2 respectively, are absent in the affected male (who is also mentally retarded), and hemizygous in his mother and in his carrier sister, who presented early in pregnancy. Analysis of chorionic villus DNA formed the basis of prenatal exclusion of choroideremia in her male fetus. In three female relatives, studied with late-labelling techniques, the deleted X was preferentially inactivated in 86–100% of cells studied. This family confirms the localisation of the choroideremia locus to within Xq13→21, and places the loci for anhidrotic ectodermal dysplasia and the X-linked immunodeficiencies outside this region.

Introduction

Choroideremia is a bilateral progressive degeneration of the choroid and retina, inherited as an X-linked recessive trait (McCulloch and McCulloch 1948; Sorsby et al. 1952; McKusick 1983). Onset of symptoms in affected males is usually in the second or third decade, with initial night blindness. Peripheral visual field defects develop, but central vision is lost late. The fundal abnormality begins with pigmentary changes, followed by atrophic changes, allowing the white sclera to show through in patches. Retinal vessels are normal. Mental retardation is not a feature of the condition. Female carriers, although normally asymptomatic, show a characteristic combination of pigmentation and depigmentation, most marked at the mid-periphery of the fundus.

No linkage has been found between the Xg blood group and choroideremia (Other 1968; Bell and McCulloch 1971). Recently Nussbaum et al. (1985) have found linkage between choroideremia and the polymorphic site DXYS1, defined by the random sequence probe pDP34. This has been localised to within Xq13→21 by in situ hybridisation (Page et al. 1984). The published linkage data show no recombination between DXYS1 and choroideremia at a LOD of 5.78. Since that publication, more family studies have confirmed this linkage ($\theta = 0.02$ at LOD 10.25; R. L. Nussbaum, personal communi-

cation), thus making the use of DXYS1 for prenatal diagnosis of choroideremia a realistic proposition. Positive LODs have also been found between choroideremia and DXS3, localised to Xq21.3 ($\theta = 0.04$ at LOD 12.32 [R. L. Nussbaum, personal communication]) and DXS11, localised to Xq24→26 ($\theta = 0$ at LOD 1.54) (Nussbaum et al. 1985; Gal et al. 1986).

We describe a kindred in which a man with classical choroideremia also suffered from mental retardation. A sister, who showed the fundoscopic changes of the carrier state for choroideremia presented 9 weeks into her first pregnancy, requesting prenatal diagnosis. For this reason, the family underwent urgent DNA analysis. The affected male was found to be deleted for the DXYS1 sequence, whilst analysis of the chorionic villus DNA from the fetus revealed a male without the deletion (Robertson et al. 1986). Family studies revealed that the choroideremia was segregating with an X chromosome rearrangement. Cytogenetic studies of the chorion sample showed that the fetus did not carry the abnormal X chromosome.

Materials and methods

The family members investigated are indicated in Fig. 1.

DNA analysis

The DNA was extracted from 10–20 ml of venous blood (with EDTA anticoagulant), and detection of restriction fragment length polymorphisms was by standard methods (Old 1986).

Cytogenetics

Chromosome preparations were obtained from peripheral blood lymphocytes by standard methods (Hungerford 1965; Moorhead et al. 1960). Late-labelling of the X chromosome was demonstrated by addition of bromodeoxyuridine (BrdU) to the cultures at a concentration of 1 mg/ml for the final 6 or 7 h of culture time. The slides were subsequently stained with Hoechst 33258 and Giemsa (Latt 1973). Late prometaphase chromosomes for high resolution banding studies were obtained by a modification of the method of Yunis (1981).

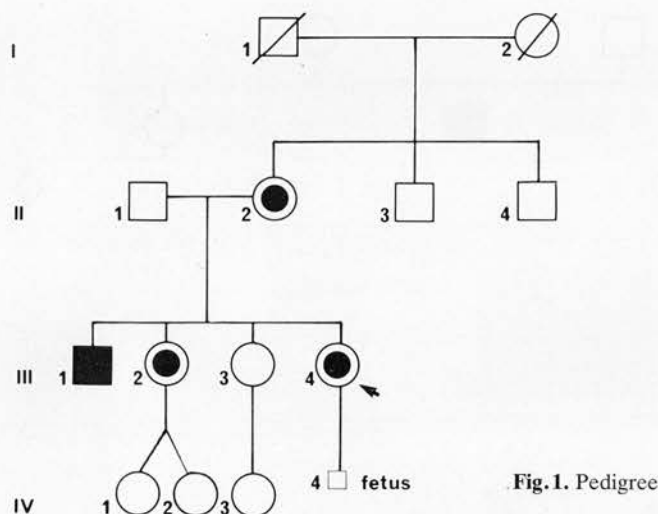


Fig. 2. Typical fundoscopic changes of carrier state, showing characteristic "salt and pepper" pigmentary changes at periphery of retina

Results

Clinical history and examination

The consultand (III.4) was referred for genetic counselling at 9 weeks gestation in her first pregnancy, knowing that she was a carrier of X-linked choroideremia. She had developed epilepsy following measles encephalitis at the age of 18 months, for which she was receiving medication, and at the age of 15 years her verbal IQ was assessed as 82. At 17 years old, her visual acuity was 6/9 (right eye), and 6/12 (left eye). Fundoscopy revealed "salt and pepper" pigmentary changes at the periphery of the retina, as typically found in choroideremia carriers (Fig. 2).

After discussion, it was decided that a chorion biopsy would be performed for fetal sexing and DNA studies, and

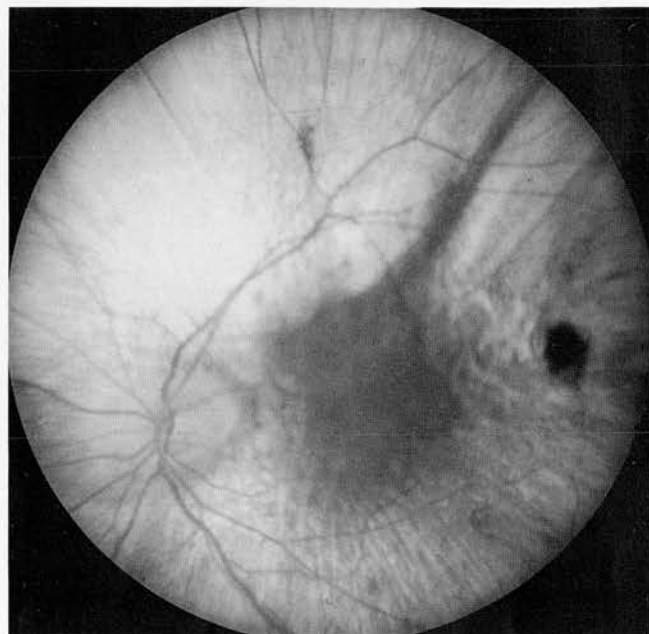


Fig. 3. Gross choroidal sclerosis with pigmentary changes, diagnostic of choroideremia, in affected individual III.1

this was done at 11 weeks of pregnancy. Once it had been demonstrated that the deletion present in the affected individual (III.1) was not present in the fetus (see below), the consultand elected to continue the pregnancy.

The affected individual (III.1) was born by normal delivery at full term, and his neonatal progress was normal. His motor developmental milestones were as follows: he sat at 5 months, crawled at 8 months, and walked at 14 months. Hair and eye color was dark, and he had no skin abnormalities. At 4 years of age, he developed a convergent squint, and was noted to be clumsy. On examination at 8 years old, he had a convergent strabismus and a high-arched palate with maxillary prognathism, and he dribbled constantly. He was clumsy and had difficulty with fine finger movements. His visual acuity was impaired, but could be corrected to 6/9 (right eye) and 6/18 (left eye). Fundoscopy showed gross choroidal sclerosis with pigmentary changes, while the discs and vessels were normal (Fig. 3), and a diagnosis of choroideremia was made. His IQ was assessed as 67 on a full-scale Wechsler test. He attended an ESN (educationally subnormal) school. He was not prone to recurrent illness or skin infections. No formal hearing assessment was performed, but he had no apparent hearing impairment.

On re-examination at 13 years of age, his eyesight had deteriorated to 6/60 in both eyes, and he had marked night blindness. He attended a sheltered workshop from his ESN school. He had never had an epileptic fit. EEG examination at this time showed a moderate diffuse abnormality of an unusual kind, with rhythmic components. His IQ was reassessed at 61 on the WISC scale, with a verbal IQ of 80, and a performance IQ of 47. At the age of 30 years he was registered blind, although he could still find his way about in daylight without assistance.

His mother, II.2, was of limited intelligence. She showed the characteristic fundoscopic changes of a choroideremia carrier, with depigmented patches in the periphery of the retina, and although her electro-oculogram was normal, the static vi-

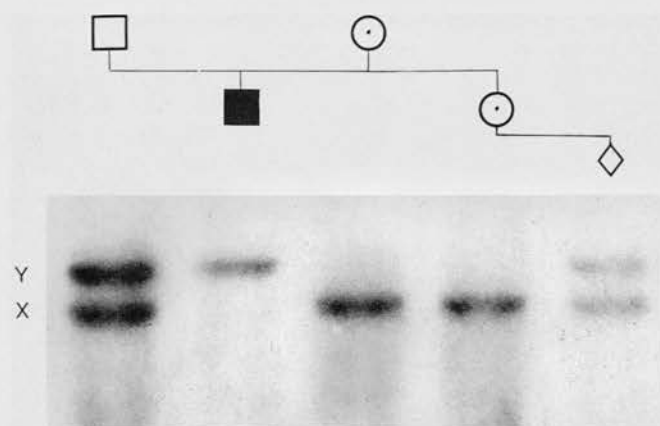


Fig. 4. A Taq1 DNA digest from the consultand (III.4), her parents (II.1 and II.2), her affected brother (III.1), and her fetus (IV.4), hybridised with the DXYS1 (pDP34) probe. As discussed in the text, the affected brother shows only the Y-specific band, the X-specific sequence being deleted. The fetus (IV.4) shows both a Y-specific and an X-specific band

sual screener showed minor defects in her central field of vision. She had several epileptic fits as a child, but not after the age of 7 years, and was not on anti-epileptic medication in adult life.

Her eldest daughter, III.2, was of normal intelligence. She was also shown to have the changes found in carriers of choroideremia on fundoscopy. She had epileptic seizures as a child, and had a few grand mal fits at 21 years of age, but since that time had no further attacks, and did not require medication. Her twin daughters (IV.1 and IV.2) have both had epileptic fits since they were 1 year of age. Twin IV.1 has severe epilepsy; twin IV.2 is more mildly affected. They were developmentally normal at 4½ years old, and both were on anti-convulsant maintenance treatment.

Individual III.3 had normal fundi, and was not thought to be a carrier of choroideremia. She was of normal intelligence, and had never had an epileptic fit.

DNA studies

DXYS1 (pDP34). This probe hybridizes to a Y-specific sequence present in a 15 kb fragment of a Taq1 digest, as well as an X-specific sequence which may show a Taq1 restriction fragment length polymorphism with 12 and 11 kb bands. Figure 4 shows an autoradiograph of the DNA from the consultand (III.4), her parents (II.1 and II.2), her affected brother (III.1), and her fetus (IV.4). The fetus is male, with a Y-specific band like those found in individuals II.1 and III.1. The affected brother has no X-specific band, indicating that there is a deletion of this sequence from his X chromosome. The fetus has an X-specific band, and therefore has not inherited the deletion. The consultand and her mother have a single X-specific band compatible with their carrying the deleted X. It was on the strength of this analysis that the consultand was reassured that the fetus had a low risk of having choroideremia (5% was quoted, given the observed linkage, and the belief that one should err on the cautious side until more data are available).

DXS3 (19.2). This probe reveals a Taq1 restriction fragment length polymorphism, the alleles being represented by an upper 5.1 kb band, or a lower doublet of 2.95 and 2.15 kb. The same filter as in Fig. 4 was re-hybridized to the 19.2 probe

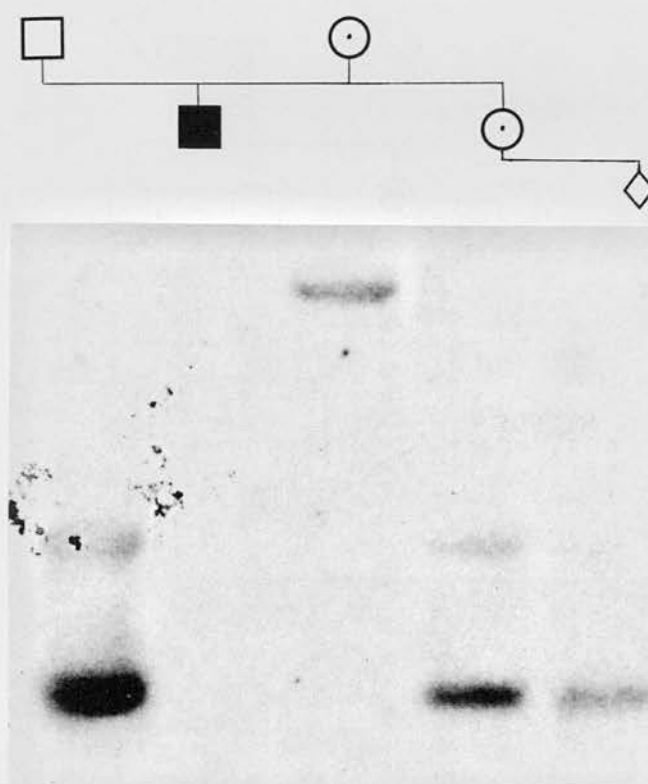


Fig. 5. The same Taq1 DNA digest filter as in Fig. 4, rehybridised to the DXS3 (19.2) probe. The Taq1 RFLP gives one allele represented by the upper (5.1 kb) band, and another by the lower doublet of 2.95 and 2.15 kb. The affected brother (III.1) shows no bands. His father (II.1) has the lower doublet, and his mother (II.2) just the upper 5.1 kb band. The consultand (III.4) appears only to have inherited the lower doublet from her father, and therefore a deleted X chromosome from her mother. The fetus (IV.4) (◇) has the lower doublet (the upper band of which is faint but clearly visible on the original)

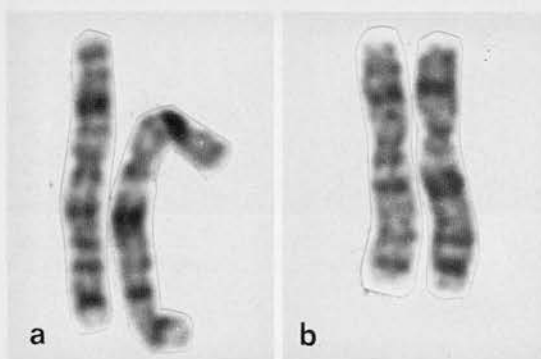


Fig. 6a, b. The X chromosome pairs from individuals IV.1 (a) and IV.2 (b). The abnormal X is on the left

(Fig. 5), and reveals that the affected brother (III.1) is deleted for this sequence also. His father (II.1) has the lower doublet, and his mother (II.2) only an upper 5.1 kb band. If the mother had been homozygous for the 5.1 kb band, then the consultand (III.4) should have had both the doublet and a 5.1 kb band. In the event, the consultand has only the doublet inherited from her father, and therefore both she and her mother carry the deletion. The fetus (IV.4) has the doublet (upper component faint), which is on the normal X chromosome originally derived from II.1.

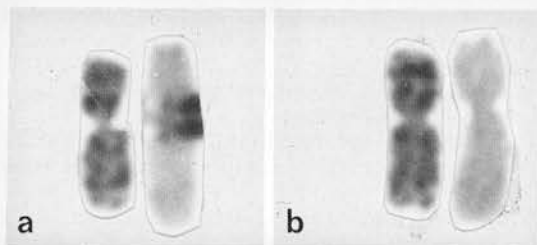


Fig. 7a, b. X chromosome pairs from two cells of the minority population, showing late replication of the normal X chromosome. The abnormal X is on the left of each pair

Cytogenetic studies

High resolution chromosome studies of the affected male showed him to have an abnormal X chromosome. The consultant (III.4) and the sister (III.2) were found to be heterozygous carriers of the same structurally abnormal X chromosome. In addition, 2/50 cells from III.2 had the karyotype 45,X, with loss of the abnormal X. The twin daughters of III.2 (IV.1 and IV.2) were both heterozygous for the abnormal chromosome (Fig. 6). III.3 had a normal female chromosome complement.

The band Xq21 is about half its normal size, due to deletion of sub-band Xq21.1. The distance from the centromere to band Xq21.3 often appears greater in the deleted chromosome than in its normal homologue. This is probably due to variation in the pericentric heterochromatic band Xq11.1, but the possibility of a more complex rearrangement in proximal Xq cannot be ruled out. Late labelling studies with BrdU showed the abnormal X to be late replicating in all 39 informative cells from III.2. In IV.1, however, 5 out of 50 cells showed the normal X to be late replicating, and the same pattern was seen in 7 out of 50 cells scored from her sister IV.2 (Fig. 7). The cytogenetic studies from the chorion villus sample (IV.4) from direct and cultured preparations showed a normal X chromosome.

Discussion

This report describes a family in which a cytogenetically abnormal X chromosome, including a deletion of the DNA sequences DXYS1 and DXS3, is segregating with choroideremia. The affected male, who demonstrates the typical ophthalmological features of choroideremia, also has moderate mental retardation, and an abnormal EEG. Four of his relations who carry the deleted X show fundal changes typical of heterozygotes for choroideremia, have all had epileptic fits, and two show mild intellectual impairment.

Given the close linkage of the choroideremia locus to DXYS1 (Nussbaum et al. 1985), it is reasonable to conclude that this locus, or the sequences concerned with its transcription, are removed or disrupted by the Xq21.1 deletion. Mental retardation is not a feature of regular X-linked choroideremia, so the intellectual impairment and EEG abnormalities in this affected male can be attributed to the loss of other X-linked gene(s) within the deletion.

It is striking that our patient has so few abnormalities, despite having such a large deletion of his X chromosome. He apparently only expresses a single X-linked disorder in addition to mild mental retardation. In particular, he shows no manifestations of anhidrotic ectodermal dysplasia, which was

recently assigned to Xq12 (MacDermot et al. 1986), or of X-linked agammaglobulinaemia, mapped to Xq21.3–22 (Kwan et al. 1986). This enables us to exclude the gene loci for these two conditions from the region of the X chromosome deleted in our patient. It is of interest that a previously described family (van den Bosch 1959) showed an association of choroideremia, mental deficiency, anhidrosis, and other features. It is tempting to speculate that this may have resulted from a deletion including the loci for choroideremia and anhidrotic ectodermal dysplasia, but unfortunately no cytogenetic studies were performed on this family. In this respect, it may be relevant that one of the deleted sequences, DXYS1, falls within a large region of the X chromosome showing homology with the Y chromosome, which may contain rather few active genes.

In the carrier women in this family, in whom X-inactivation studies were performed in lymphocytes, the abnormal X was inactivated in 86–100% of cells scored. In almost all reported cases of deletions of the X chromosome, the structurally abnormal X chromosome was late-replicating in lymphocytes and fibroblasts (Francke 1984). Exceptions to this observed pattern are reports of two patients with Turner syndrome who had terminal deletions of Xp (Petrinelli et al. 1978; Kaosaar and Mikkelsaar 1980), where in a small proportion of lymphocytes the deleted X chromosome replicated early; the mildly retarded woman with an Xp deletion described by Francke (1984); and the mother of the boy described by Ferguson-Smith et al. (1982), in both of whom late-replication of the X was random. Similarly, although late-labelling of the normal X in female carriers of balanced X/autosome translocations is the general rule, late replication of the translocated X chromosome in a proportion of cells has been described (Mattei et al. 1982). This bias in inactivation has often been ascribed to cell selection in favor of the most balanced karyotype. However, it is difficult to argue that nullisomy for this X deletion in cells in which the normal X is inactivated would be cell-lethal and lead to selection in favor of cells in which the normal X is active, because the deletion has not been lethal to the affected male.

Individual III.4 had fundoscopic changes of the carrier status for choroideremia, despite the fact that in all her scored lymphocytes the deleted X chromosome was late-labelled. However, because the relationship between late-labelling and X inactivation is unclear, and differential inactivation in different tissues has been described (Hellkuhl et al. 1982), we cannot make assumptions about which X chromosome was active in her retinal tissues. The deletion could, alternatively, affect development in the early stage of embryonic life at which both X chromosomes are active, causing abnormalities of the fundus which become apparent in adult life.

The chromosome abnormality and DNA deletion we describe are segregating with choroideremia in this family. In view of the linkage previously demonstrated between choroideremia and DXYS1, we propose that the deletion was causally related to the disorder, and that the gene locus for choroideremia lies within the region defined by the deletion in this family. The gene locus for X-linked ectodermal dysplasia, by the same argument, must lie outside the region of the deletion.

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